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Iowa State University

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Genistein's effects on lytic activity and intracellular tyrosine
phosphorylation of natural killer cells

by

Kim Annette Downard

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Immunobiology

Major Professor: Joan E. Cunnick

Iowa State University

Ames, Iowa

2000

Graduate College
Iowa State University

This is to certify that the Master's thesis of
Kim Annette Downard
has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

TABLE OF CONTENTS

ABSTRACT	iv
INTRODUCTION	1
LITERATURE REVIEW	3
MATERIALS AND METHODS	30
RESULTS	37
DISCUSSION	62
REFERENCES	65
ACKNOWLEDGEMENTS	75

ABSTRACT

Genistein, an isoflavone, specifically inhibits protein tyrosine kinases (PTK). In low concentrations, genistein enhances natural killer (NK) cell lytic activity of peripheral blood leukocytes (PBL) in a dose dependent manner. The following study was designed to investigate if the enhancement of lytic activity was associated with the modulation of PTK's by genistein. A flow cytometric assay was developed to monitor phosphorylation of intracellular tyrosine residues in a pure population of NK cells (NK 3.3) in parallel with a chromium release assay for lytic activity. The flow cytometric assay used an anti- phosphotyrosine-FITC conjugated antibody in conjunction with the phosphatase inhibitor, pervanadate. Genistein at concentrations of 0.1-10 μ M was found to inhibit baseline phosphorylation and phosphorylation in the presence of pervanadate of activated NK3.3 cells. However, while tyrosine phosphorylation was inhibited, the lytic ability of NK 3.3 cells against K562 cells was not affected and showed a slight enhancement of lytic activity. Genistein was observed to enhance the lytic ability of rested NK3.3 cells as well as a corresponding enhancement of intracellular phosphotyrosine residues. Since tyrosine phosphorylation provides an early and requisite signal for NK cell lytic activity, these results suggest that another pathway (non-phosphotyrosine) is utilized during activation of lytic activity. Thus, genistein does not appear to enhance lytic activity of NK 3.3 cells by modulation of their PTK's.

INTRODUCTION

The lower incidence of breast, colon, and prostate cancer in Asian countries brought attention to the differences in diet between Asian and Western cultures, particularly the consumption of soy-containing foods (36). The anti-cancer effects are thought to be due to isoflavones in soy, principally genistein. Use of in vivo (animal) models, demonstrate that soy products containing genistein in the diet significantly reduce the risk of cancer. Use of in vitro models, demonstrate that genistein inhibits the proliferation of human tumor cell lines (5). Previous in vitro studies in our lab demonstrated enhancement of lytic activity of harvested human NK cells against human tumor cells in the presence of low concentrations of genistein ($< 1 \mu\text{M}$). These effects may be related to regulation of NK cells that are involved in killing tumor cells and virally transformed cells.

Tyrosine phosphorylation has been found to be an early and necessary signal for lytic activity in natural killer (NK) cells (20). Genistein, an isoflavone, is a specific inhibitor of protein tyrosine kinases (PTKs) and can be used as a tool for understanding the role PTKs play in the activation of cytotoxicity (2). Previous studies have shown that genistein ($10 \mu\text{g/ml}$ or $37 \mu\text{M}$) inhibits the src protein tyrosine kinase p56^{lck} in "NK-rich" cells exposed to IL-2 with a resultant decrease in cytotoxic activity against K562 cells (39). Thus, biological studies have shown that genistein can enhance or inhibit NK cytotoxicity depending on the assay conditions used.

The purpose of this research was to ask if low concentrations of genistein had a direct effect on natural killer cell lytic activity and if the observed cytobiological effects of genistein were related to changes in signal transduction, particularly protein tyrosine kinases (PTKs). Initial studies used NK cells harvested from peripheral blood leukocytes. When it was observed that genistein had an effect on lytic activity, a pure NK cell line was used to study the direct results of genistein on NK cells. The NK3.3 cell line was used as a model system so that a pure population of natural killer cells would define direct results of genistein on those cells. Intracellular levels of phosphorylated tyrosine residues after treatment with low doses of genistein were examined by flow cytometry (21, 24). Lytic activity was assayed in parallel by a chromium release assay (12). Together these studies attempt to define a relationship between enhanced lytic function and intracellular tyrosine phosphorylation due to genistein.

LITERATURE REVIEW

Innate Immunity

The immune system is divided into two branches. There is acquired (adaptive) immunity that specifically recognizes and eliminates that which is foreign. The second branch is innate (natural) immunity, which is the basic resistance to disease one is born with. Contrary to some beliefs, the innate immune system is not some left over relic from evolution nor is it the “containment crew” until adaptive immunity “kicks in”. Rather adaptive immunity owes its existence to innate immunity. Innate immunity is composed of anatomic barriers, phagocytic cells, complement, and natural killer cells. The last three components of the innate immune system generate signals that activate and even direct the type of effector response of acquired immunity (35, 46).

Neutrophils and macrophages are two key phagocytic cells of innate immunity. Inflammation is the hallmark of an innate immune response and it also activates neutrophils and macrophages (46). Neutrophils are the most numerous of granulocytes found in the blood. Macrophages are derived from monocytes circulating in the blood that migrate into tissue and become tissue specific macrophages (28). Both cell types recognize conserved structures found on microbes called PAMPS for pathogen-associated molecular patterns and components of the complement system (35, 46). In addition, both have complement receptors and Fc receptors in order to recognize organisms covered with C3b, an active fragment of complement component C3 or antibody, a process called

opsonization. Neutrophils and macrophages engulf microbes. The granules of neutrophils contain lytic enzymes and bactericidal substances that fuse with a phagosome to digest infectious material (28). Neutrophil granules also induce the release of biological response modifiers, cytokines and chemokines, by macrophages (46). Macrophages, after internalizing infectious material, destroy them by the creation of a phagolysosome that contains hydrogen peroxide, peroxidase, lysozyme, and other hydrolytic enzymes, which digest the material. The digestive fragments (antigens) are brought to the surface of the macrophage where they can be presented to T-cells (28, 47). Macrophages binding to infectious material also stimulate the release of cytokines and chemokines.

Cytokines released by phagocytic cells are able to activate the complement cascade. Complement is a group of serum proteins involved in inflammation, activation of macrophages, and participates in an enzymatic cascade that culminates in lysis of cell membranes (47). Complement and phagocytic cells have a symbiotic relationship. Complement activation induces chemotaxis and opsonization, which generates the participation of macrophages. Macrophages produce cytokines that stimulate the liver to produce acute phase proteins, C-reactive protein and mannan-binding lectin (MBP) that activate complement by different pathways. C-reactive protein does this by binding to and activating complement component one, much like the initiation of the classic pathway by antibody binding. MBP recognizes and binds to mannose found on bacterial cell walls to activate complement by the lectin pathway. This reaction substitutes for the activation of the first component of complement.

Phagocytic cells and complement are efficient for dealing with extracellular bacterial infections but they cannot reach pathogens (viruses as an example) that reside inside of cells. Natural killer cells have the intrinsic ability to lyse virally infected cells and tumor cells particularly hemopoietic tumors. NK cells play a role in innate immunity as effector cells and a source of cytokines that modulate other immune cell activities (45).

Natural Killer Cells

Natural killer (NK) cells can be found in earthworms, sharks, common fish, amphibians, reptiles, birds, and mammals. In humans, NK cells are the third major class of lymphocytes and comprise 10 -15% of the lymphocyte population in the blood, liver, and especially in the spleen (red pulp), but are rarely found in lymph nodes. NK cells are thymic independent, do not undergo immunoglobulin (Ig) or T cell receptor (TCR) gene rearrangement, have no classic antigen binding receptors, and no CD3. NK cells have non-specific cytotoxicity and do not require antigenic stimulation or MHC class restricted presentation of antigen. The developmental lineage of NK cells is unknown, however there are two theories as to their origins. One is that NK cells develop from a distinct lineage from that of T-cells (since SCID mice who have no T-cells still have NK cells) or second, both NK and T cells originate from the same early progenitor cell before rearrangement of TCR genes since NK cells do share some markers with T-cells. Morphologically, NK cells are referred to as large granular lymphocytes (LGL) due to having a high cytoplasm to nucleus ratio, a kidney shaped nucleus, and large azurophilic granules (28). NK cells

can be activated (to kill targets) by cytokines or cell-to-cell contact (44). Definitive cell surface markers for NK cells are CD16 and CD56. CD16 is a Fc γ RIII receptor found as a Type I transmembrane protein on NK cells. CD16 belongs to the Ig superfamily and mediates binding to IgG coated particles. CD56 also is a Type I transmembrane protein, member of the Ig superfamily, and is an isoform of N-CAM (neural cell adhesion molecule) called a hemophilic adhesion molecule. CD2 (LFA-2) is another marker found on the NK cell surface. CD2 is also found on T-cells and is sometimes called the E-rosette receptor. It is a Type I transmembrane protein of the Ig superfamily, an adhesion molecule whose ligand is CD58 that is found on many cell types. In the case of T-cells, CD2 serves as an accessory molecule to promote binding of cytotoxic T-cells to their targets (23). CD57, also known as HNK-1, is an oligosaccharide present on NK cells with no known function. NK cells express the β and γ signal transducing subunits of the IL-2 receptor but not the α subunit required for high affinity binding of IL-2 to the receptor. When expressed, IL-2R α reduces the amount of IL-2 needed for activation and growth. Since there is no IL-2R α expressed after stimulation with IL-2, NK cells, unlike T cells when activated with IL-2, cannot reduce the amount of IL-2 needed to maintain activation (1).

When stimulated by high concentrations of IL-2, NK cells proliferate and lytic activity is enhanced. This enhancement is a consequence of IL-2 activation to transform NK cells to lymphokine activated killer (LAK) cells. LAK cells have enhanced cytotoxic ability and a broader range of target specificity. This means that LAK cells, unlike NK cells, can kill a wider variety of tumor cells and even normal cell types like epithelial cells.

There are two pathways that NK cells utilize for cytolytic activity: antibody dependent cellular cytotoxicity (ADCC) by CD16 and direct cell-mediated cytotoxicity. With ADCC, the target cell is coated with specific IgG to which the low affinity $Fc\gamma$ receptor, CD16, binds. This binding engages the pathway to lytic activity while also activating NK cells to synthesize and secrete cytokines such as TNF and $IFN-\gamma$. Since CD16 is a low affinity receptor, it can only bind to aggregate but not monomeric IgG (1).

Antibody independent lysis is not as well understood as that for ADCC since no known definitive activating receptor for NK cells or ligand on target cells has been identified. There are several theories as to how this pathway may be activated. One is that direct cell-mediated cell lysis is triggered by an unidentified surface NK receptor to an unknown structure on a malignant or virally infected cell (1, 20). Another is that target cells lack expression of a normal protective molecule. The identification of the target molecule not being encoded in MHC class I or that lack of expression of MHC class I induces lysis. The latter theory is supported by the fact that virally infected cells and tumor cells have decreased MHC I expression.

There is also the two-receptor model theory, which postulates regulation of NK lytic activity through inhibition of lysis. That is, the NK cell receives signals from two different receptors. One receptor binds to ligands on the target cell that are possibly carbohydrates, this is the signal to kill. This signal is prevented by a second receptor that binds to certain alleles of MHC class I. This negative signal overrides the

positive signal. Low MHC class I expression would result in a reduction of the negative signal so that the positive signal would be greater and result in killing (1, 28, 44).

Though no known activating receptor has been characterized for NK cells this has not been the case for inhibitory receptors found on NK cells. Inhibitory receptors have been found on macrophages, mast cells, and killer cells (13). There are inhibitory receptors that specifically bind to MHC class I molecules. On human NK cells there are two groups of inhibitory receptors. There are Type I transmembrane proteins with immunoglobulin domains and long cytoplasmic tails (76,84, or 95 amino acids long) called killer inhibitory receptors (KIRs). There are two families of KIRs, the p58 and p70 inhibitory receptors (18, 25). Three human NK KIRs have been characterized. GL183 and EB6 (p58s) each have two Ig domains and recognize HLA-C alleles at positions 77 and 80 on the $\alpha 1$ domain of the MHC class I molecule. NKB1 (p70) has three Ig domains and recognizes the Bw4 epitope on HLA-B at positions 77-83 on the $\alpha 1$ domain of the MHC class I molecule (56). In addition there is also a homodimer of p70 called p140 that can recognize HLA-A alleles (13). Human KIR recognition of polymorphic determinates of HLA molecules has been found to be limited to the carboxy-terminal of the $\alpha 1$ domain of MHC class I (56). A second group of human inhibitory receptors are Type II transmembrane proteins with C type lectin extracellular domains. One type of this receptor, CD94/NKG2, exists as a heterodimer that also binds MHC class I (13, 25, 29). As CD94/NKG2 has C-lectin domains, it may be able to bind to a common carbohydrate in different MHC class I alleles and would enable this receptor to have a broader

binding capacity allowing it to recognize remaining HLA-B and HLA-A alleles not recognized by the Type I KIRs. Both classes of inhibitory receptors have in common that they recognize and bind to MHC class I molecules but also contain two immunoreceptor tyrosine inhibitory motifs (ITIMs) separated by 26-28 amino acids in their long cytoplasmic tails (18, 37).

It is hypothesized that NK inhibitory receptors prevent cell activation in certain conditions, times, or locations to protect sensitive areas. It is suggested that inhibitory receptors prevent harmful activity of NK cells while the cells mature and migrate where their presence is required (13). However, binding of KIRs does not lead to cell death or long-lived anergy. The identification of KIRs provides evidence to validate the two-receptor theory for activation.

NK inhibitory receptors override activation signals to prevent lysis. When KIRs bind MHC class I ligand, downstream signaling events responsible for generating inositol-1, 4,5-trisphosphate (IP3) and increasing intracellular calcium are shut down and there is a partial disruption of tyrosine phosphorylation (56). Upon engagement of KIR to MHC class I, tyrosine residues in the ITIMs are phosphorylated by a src family member (lck and lyn, but not fyn) (7, 14). This phosphorylation in turn recruits and activates the tyrosine phosphatase, SHP-1 (14, 15). SHP-1 appears to dephosphorylate an early substrate that is found in the activation pathway. A possible candidate for that substrate could be the adapter protein pp36 that associates with phospholipase C gamma (PLC γ) or another adapter protein called Grb2. Grb2 and pp36 control PLC γ enzymatic activity (55). For inhibition by KIRs, there must also be co-ligation with an activating receptor. This would suggest that

KIRs and activating receptors would need to be in close proximity to each other (13).

There are a group of KIRs with short cytoplasmic tails (39 amino acids long) that have no ITIMs, and have a charged lysine in the transmembrane domain indicating they have the ability to associate with signal transducing molecules. These receptors are known as killer activating receptors (KARs) and have been proposed to be activating receptors for NK cells (18).

Mechanism of Target Cell Lysis

The process of target cell lysis by NK cells is the same whether initiated by ADCC or the Ab-independent pathway and is similar to that used by cytotoxic T lymphocytes. This process includes granule exocytosis and initiation of DNA fragmentation and apoptosis. As they contain large granules at all times in the cytoplasm, NK cells are always constitutively cytotoxic unlike T-cells which must be stimulated before granules appear. The cytotoxic process is initiated by the recognition of target through cell-to-cell contact. The contact occurs through CD16 on an NK cell binding to IgG on a target cell or the binding of the unknown receptor and ligand for Ab-independent lysis. Once adherence takes place, a signal is sent to activate the lytic pathway. Degranulation of the NK cell occurs at the site of contact. NK granules contain perforin, granzymes (neutral serine proteases), proteoglycan, and cytotoxins. These substances deliver the "lethal hit" to the target cell. Perforin is a monomeric pore forming protein that requires the presence of calcium so it can bind to the target membrane and form a pore. After entering the target cell, granzymes bind to substrates that are involved with apoptosis or can be transported

to the nucleus where they can directly bind and activate death substrates. Cytokines such as TNF- α enter the target to further damage the target cell integrity. So unlike the “true” lysis seen with complement, NK cells activate an endogenous apoptotic pathway with granzymes leading to cell death. Proteoglycan (chondroitin sulphate A) protects the NK cell from self-destruction. After an NK cell delivers its “lethal” hit, it can be “recycled” to lyse other targets, become inactive, or undergo apoptosis (1, 28, 31, 44, 47, 50).

Natural Killer Cell Functions

The two major roles of NK cells are immune surveillance against tumor cells and virally infected cells and the release of cytokines (specifically IFN- γ) to activate macrophages and T lymphocytes (44). NK cells can inhibit proliferation of tumor cells and blood borne metastasizing tumor cells by their characteristic “natural” killing (42). Tumor cells have decreased MHC class I expression especially those tumors mediated by oncogenic viruses. While reduction of MHC class I compromises cytotoxic T lymphocyte activity, it seems to be one requirement for recognition and lysis by NK cells (1, 28). Tumor cells may also express specific surface proteins that NK cells recognize (1). NK cells can lyse tumor cells coated with antibody by ADCC and this killing is unaffected by the presence or absence of MHC class I. Chediak-Higashi syndrome and the animal model of this disease called beige mouse readily demonstrates the importance of NK cells in tumor immunity. In Chediak-Higashi patients, NK cell lytic activity is impaired due to an abnormality in

the granules that contain granzymes. Patients experience recurrent infections and an aggressive but nonmalignant infiltration of organs by lymphoid cells (1, 28).

NK cells have other functions as they serve to control infections caused by viruses, parasites, fungi, and bacteria. NK cell produced cytokines are of more importance than NK lytic activity in controlling bacterial infections (53). Bacterial infections cause inflammatory signals that attract NK cells. NK cells can destroy extracellular bacteria via ADCC by binding to opsonizing IgG bound to bacteria. Macrophages containing intracellular bacteria are stimulated to produce IL-12, which activates NK cells. In response to this activation, NK cells produce cytokines (IFN- γ) to recruit other immune effectors involved with inflammatory responses (1, 22, 42). NK cells are not known to directly kill parasite-infected cells or parasites. NK cells do contribute to early opposition of parasitic infections by producing IFN- γ to stimulate macrophage activity and influence a Th1 response to direct an adaptive immune response to parasitic infections (53). Viral infections directly activate the production of Type I IFN by the infected cells and down regulate MHC class I expression, all which serve to enhance and stimulate NK lytic function (1).

NK cells function in immune regulation and the pathology of disease states. NK cells participate in immune regulation through the production of cytokines. Some of the immunoregulatory events that NK cell cytokines participate in are innate immune responses (chemokines, IL-1), regulation of lymphocyte activation, growth, and differentiation (IL-2, IL-4), immune directed inflammatory reactions (IFN- γ , IL-12), and hematopoiesis (IL-3, GM-CSF). NK cells have roles in autoimmune disorders and graft vs. host disease (GVHD). When antibody binds to self-antigen as in

autoimmune diseases, CD16 on NK cells binds to IgG, which activates lysis by ADCC. GVHD occurs when grafted effector cells mount a rejection response to the host resulting in epithelial cell necrosis. NK cells cannot recognize allotype antigens but transplanted T helper cells do and are stimulated to produce IL-2. In response to IL-2, NK cells differentiate into LAKs that are able to lyse host epithelial cells (1).

NK3.3 Cells

Most studies of NK cells have been performed using partially purified cells from humans or animals since the method of harvesting NK cells by density gradient centrifugation with Ficoll-Hypaque does not yield a pure population. However, research on NK cell biology using partially purified NK cells was unable to rule out cytotoxicity due to other immune cells, eliminate possible cross-talk between other cell types, or solve the problem of collecting an adequate quantity of pure NK cells. A specific NK cell line was needed to eliminate these questions and link results specifically to NK cells. Dr. Jacki Kornbluth, St. Louis Univ., St. Louis, MO developed such a cell line.

Dr. Kornbluth developed the NK3.3 line, a human cell line, from a primary mixed lymphocyte culture (MLC). This was accomplished by harvesting peripheral blood leukocytes (PBL) by density gradient centrifugation from heparinized blood provided by two healthy donors. The responder PBLs were incubated with an equal number of irradiated stimulator PBLs for six days, 37⁰ C, and 5% CO₂ (6 day MLC). From the primary culture, 1 - 2.5 x 10⁵ cells were plated in 1 ml of 0.45% agarose containing RPMI-1640 complete media, 20% human serum, and 30% IL-2 conditioned media.

This mixture was plated in a 24 well plate at 1 ml per well and incubated for another 6 days at 37° C. Individual colonies were removed from the agarose and expanded in microtitier plate wells with liquid media containing RPMI-1640 complete media, 15% human serum, and 20% IL-2 conditioned media. Colony 3 after being maintained in IL-2 conditioned media for one month was split into 8 separate wells. The NK3.3 cell line is a subculture from one of the individual colonies (27).

NK3.3 cells are morphologically and cytochemically similar to LGLs. NK3.3 has a high cytoplasm to nucleus ratio, azurophilic granules in the cytoplasm, and is capable of strong direct cell contact lysis but has weak lysis by ADCC. The NK3.3 target specificity was established using cold target inhibition assays. The NK3.3 cells can lyse the following NK susceptible lysis cell lines in order of strongest to weakest cytotoxicity: K562, MOLT-4, BUC, HSB-2, CEM, and DAUDI. NK3.3 is an IL-2 dependent cell line (27). Phenotypically NK 3.3 is CD2+ (E-rosette receptor), CD3- (accessory T-cell receptor), CD4/8- (T-helper and T-cytotoxic markers), CD16+ (FcγRIII receptor), CD38+ (ADP-ribosyl cyclase), CD45+ (tyrosine phosphatase), CD56+ (adhesion molecule), HLA-DR+, -DP+, -DQ+, and no TCR (17).

The NK3.3 cells have been used to observe what factors affect cytotoxicity. As NK3.3 cells are constitutively active due to IL-2 dependence, they were used to show that IL-2 activated NK cells natural cytotoxicity is reversibly inhibited by extracellular nucleotides with increasing negative charge in a manner similar to non-stimulated NK cells. However, when using the adenine nucleotide analog, 5'-p(flurosulfonyl)benzoyl adenosine (5' FSBA), cytotoxicity was found to be irreversibly inhibited. This information can be used to discover the role that

nucleotide-binding proteins have on extracellular regulation of cytotoxicity of NK cells (19). The natural killer tumor recognition (NK-TR) protein was found to be necessary in the activation of direct cell contact lysis by NK3.3 cells (40). Glucocorticoid suppression of NK cytotoxicity was investigated using NK3.3 cells. Through the use of micromolar concentrations (1-10 μ M) of cortisol with NK3.3 cells, the following molecular mechanisms contributing to suppression were observed: granzyme A synthesis suppressed, down regulation of adhesion molecules, reduced ability to form conjugates with K562 cells, and lytic activity abolished (69).

The NK3.3 cells have been used in various research studies to provide explanations for biochemical and molecular events that mediate signaling events related to NK cell functions. Cone, et al., (17), used NK3.3 cells to demonstrate that the src protein tyrosine kinase p56^{lck} was linked to CD16 and was a signal transducer for ADCC. The NK3.3 cells have been used to show that NK cells respond to IL-2 and IL-12 to produce IFN- γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) by using separate and distinct pathways to induce IFN- γ and GM-CSF gene expression (64). An association between the phosphatase CD45 and p56^{lck} kinase and its effects on IFN- γ production has been demonstrated with the NK3.3 cells (61). Stimulation of NK3.3 cells with IL-12 and IL-2 was shown to result in phosphorylation of distinct but related Janus (JAK) family tyrosine kinases. IL-12 stimulated phosphorylation of Tyk2 and JAK2 whereas JAK1 and JAK3 were phosphorylated in response to IL-2. This research demonstrated that even though these two cytokines work together synergistically both use separate signaling pathways (4). The NK3.3 cells were used to establish a signaling pathway linking

CD2 mediated granular exocytosis with p72^{syk} kinase, the adapter protein Shc, and phosphatidylinositol 3-kinase (PI3K) activity (54). NK3.3 has been used to analyze the molecular events that occur in response to stimulation with IL-2, IL-12, and IFN- γ on JAK-STAT (signal transducer and activators of transcription) signaling pathways (65).

NK3.3 cells were used to see if PI3-kinase (p85 α) physically associated with ITIMS (immunoreceptor tyrosine-based inhibitory motif) found in KIR (killer cell inhibitory receptors) cytoplasmic tails in NK cells. Cross-linking of KIRS on NK3.3 cells by GL183 was performed to determine whether this would lead to recruitment of p85 α . KIRs were immunoprecipitated from exponentially growing NK3.3 cells with GL183 (anti-KIR, IgG) and blotting with p85 α antibody detected p85's presence. It was found that cross-linking by GL183 did induce association between KIR and p85 α (34).

In parallel, KIR cross-linking by GL183 was examined for increased lipid kinase activity of PI3-K. In these assays, p85 α was immunoprecipitated from NK3.3 cells that were cross-linked with GL183 and p85 α was assayed for its ability to transfer ³²P to PI4-phosphate. It was observed that PI3-K activity paralleled the recruitment of p85 α to KIR cytoplasmic tails (34).

PI3-K is upstream of other signaling intermediates. It was observed that another consequence of KIR cross-linking by GL183 was the activation of AKT by PI3-K. AKT is a serine/threonine kinase credited with anti-apoptotic activity. This result suggested that KIRs could also convey positive signals for growth and survival.

Activation of AKT would protect NK cells from apoptosis. This means that not only could KIR participate in inhibitory signaling but also positive signaling (34).

NK3.3 cells have been used to examine cell cycle progression in NK cells. Yamauchi and Bloom (63) used NK3.3 cells to define the intracellular events that could be induced by the absence of a reducing environment. It had been previously found that IL-2 stimulated functions were subjected to downstream redox regulation. NK3.3 cells cultured in a thiol-deficient, IL-2 containing media failed to proliferate. This was due to NK3.3 cells being arrested in the G1 phase and unable to move on to the DNA synthesis phase of the cell cycle. Thus, for IL-2 stimulated NK3.3 cells to proliferate and function they require a reducing environment for normal cell cycle progression.

Overall, NK3.3 cells have been used extensively in defining NK cell activation, regulation, signal transduction mechanisms, and should prove useful in the evaluation of new biological response modifiers.

Biological Response Modifiers

Biological response modifiers are substances made from living organisms that can produce an action or change a condition. Cytokines are one example of a biological response modifier. Cytokines are low molecular weight soluble proteins made and secreted by cells that can influence the function of other cells expressing specific surface receptors. Cytokine receptor binding triggers signal transduction pathways to alter gene expression (28, 44). Lymphocytes are an important source of

cytokines as well as macrophages. Cytokines have functions in the immune system but also have roles in other systems such as developmental biology (23). The term cytokine is used for a large group of diverse molecules. However, cytokines can be categorized into four principal groups by their actions. The first group are those cytokines that mediate innate immunity and include the type I interferons (IFNs) that are the first line of defense for viral infections as well the cytokines which mediate inflammatory reactions (TNF, IL-1, IL-6, chemokines). The second group of cytokines are those produced by antigen stimulated T helper cells and act to regulate the growth, activation, and differentiation of T and B cells (IL-2, IL-4, TGF β). The third group are the cytokines that are produced by activated T helper and T cytotoxic cells and activate inflammatory leukocytes (INF- γ , lymphotoxin, IL-10, IL-5, and IL-12). The fourth group of cytokines are called colony-stimulating factors. Bone marrow stromal cells and T cells produce these cytokines in order to stimulate the growth and differentiation of bone marrow progenitor cells (1).

Cytokines are able mediate a broad range of activities in the immune system. NK cells are regulated by specific cytokines and also secrete specific cytokines in response to a stimulus.

The most potent activators of NK cells are IL-2, IL-12, and INF- γ . IL-12 was formerly know as NK stimulating factor and is a protective cytokine in infections involving intracellular pathogens. IL-12 activates, induces proliferation, and enhances the secretion of INF- γ and cytotoxicity of NK cells (23, 47). IL-12 works best in synergy with IL-2 to activate NK cells (44). IL-2 was previously known as T cell growth factor (47). The major function of IL-2 is to induce immune responses. IL-

IL-2 is made primarily by T helper cells after stimulation by MHC class II and antigen stimulation. IL-2's effect on NK cells is to increase lytic activity, induce clonal expansion, and generate differentiation to LAKs (23, 44). Interferon gamma (IFN- γ) is best known as a major activating factor for macrophages as it increases their metabolism, phagocytic and killing activities, especially those involving intracellular pathogens. IFN- γ also increases MHC class I expression and induces MHC class II expression. IFN- γ is able to induce differentiation of B cells and NK cells and affect T cell development. It can increase NK cell adherence to target cells and activate and increase the rate of target lysis (23, 44).

To a lesser degree the following cytokines are also known to activate NK cells: IL-1, IL-15, and Type I interferons (α and β) (28). IL-15 has similar effects to IL-2 as it increases proliferation and effector function of NK cells. IL-1 is a T cell activator and enhances NK cell activity (23, 28). Type I interferons (α and β) are secreted by virally infected cells and stimulate NK cells. This activity of NK cells by IFN- α and IFN- β is the first line of defense to viral and some bacterial infections until T cytotoxic cells differentiate into cytotoxic T lymphocytes (28).

Recent studies with chemokines (chemotactic cytokines) have shown that they too can increase cytotoxicity and direct the migration of NK cells to sites of inflammation and tumor cells (32). Chemokines are low molecular weight heparin binding polypeptides that are released at inflammatory sites and initiate the migration of immune cells. Chemokines are divided into families by the location of cysteine residues in the NH2 terminus: CXC (α), CC (β), C (γ), and CX₃C (δ).

Chemokines also mediate expression and/or adhesiveness of leukocyte integrins and are specific for cell types. Chemokine binding to its receptor triggers an activating signal that is mediated by heterotrimeric G-proteins that are associated with the receptor. The signal induced by chemokine binding causes a conformational change in the integrin molecules on the cell membrane. This increases the affinity of integrins for adhesion molecules. Cells will migrate in the direction of high concentrations of chemokines (28, 47).

NK cells do not normally recirculate between blood and lymphoid tissues but they can after the administration of biological response modifiers such as chemokines. LAK cells do not migrate towards tumor sites (32). Chemokines induce chemotaxis of NK cells and mobilization of their intracellular calcium, which is a common second messenger in signaling events. The CC (β) chemokines in particular induce granule exocytosis of NK cells therefore activating and enhancing their cytotoxicity. Research with chemokines and NK cells suggests that chemokine activated NK cells are better suited for tumor therapy than IL-2 activated LAK cells. NK cells have better migratory behavior (extravasate into tissues) than LAK cells which do not respond to chemokines and do not migrate to tumor sites. Moreover, to maintain LAK cells in vivo, a continuous infusion of IL-2 is required which can be toxic in high doses; this is not the case with chemokines. Chemokines attract and activate NK cells at sites of viral infection to lyse virally infected cells. Chemokines also have the ability to inhibit replication of HIV-1 infected cells by binding to chemokine co-receptors. Taken together these actions suggest that chemokines can be used as tools to study signal transduction pathways in NK cells (32).

NK cells must be activated to synthesize and secrete cytokines. The dominant cytokine that NK cells are known to produce is IFN- γ . IFN- γ is also called immune or type II interferon and is a cytokine that regulates immune-mediated inflammation. Like type I interferons, IFN- γ is antiviral and antiproliferative. However, IFN- γ binds to a different cell surface receptor and also has immunoregulatory functions affecting primarily macrophage activation (1).

Activated NK cells also produce tumor necrosis factor- α (TNF- α) and granulocyte-macrophage colony stimulating factor (GM-CSF) (55). TNF- α also referred to as cachectin, is a prominent inflammatory cytokine with a wide range of activities. Early studies of this cytokine showed that it could kill tumor cells in tissue. TNF- α is an endogenous pyrogen, induces leukocytosis, enhances endothelial adhesiveness, and enhances the production of other cytokines. GM-CSF is a cytokine that is important for the development of myeloid lineage-specific bone marrow stem cells. GM-CSF sends strong signals to induce TH 1 differentiation and response (23).

Activated NK cells secrete the following interleukins: IL-1, IL-2, IL-3, IL-4, and IL-12 (28, 42, 47). The primary function of IL-1 is that of a mediator cytokine of a host inflammatory response in innate immunity (1). IL-1 stimulates T and B cells, travels to the brain to induce fever and augment corticosteroid release, and induces the production of acute phase proteins in the liver. An example of an inflammatory response that IL-1 participates in is the production of prostaglandins and degradative enzymes (collagenase) that are involved in cartilage and bone destruction (47).

With the production of IL-2 and IL-12, one should note that cytokines, like hormones, demonstrate autocrine action, meaning that the cell that produces the cytokine can also be the target of the cytokine. These two cytokines when working together are potent effectors of NK cell and CTL cytotoxic activity. IL-12 stimulates naive T cells to TH 1 response while IL-2 contributes to the growth and differentiation of B cells (1, 47).

IL-3 is also known as the multilineage colony-stimulating factor (multi-CSF) (1). This interleukin works as a haemopoietic growth factor by stimulating the proliferation and differentiation of bone marrow pluripotent stem cells and lineage committed precursors (23). IL-4 is a B cell growth factor cytokine that leads to activation and differentiation of B cells. IL-4 drives the immune response towards humoral and antibody production particularly IgG1 and IgE. IL-4 is a growth factor for mast cells and works in synergy with IL-3 to stimulate mast cell proliferation. IL-4 can also up regulate the expression of specific adhesion molecules (example VCAM-1). IL-4 is a growth and activation factor of T cells directing them towards a TH 2 response and an inhibition of IL-1 and TNF (1, 23, 47).

NK cells can be stimulated by and produce cytokines to biologically modify the response of the immune system. However, biological response modifiers are perhaps not the only substances to effect on NK cell activity. This research will examine genistein, a phytochemical, which can modify the cytolytic response of NK cells against target cells.

Genistein

Phytochemicals are plant derived bioactive non-nutrients (48). One group within the phytochemicals is the phytoestrogens that have nonsteroidal structure that can act as estrogen receptor agonist or antagonists (48). Two main classes of phytoestrogens are lignans and isoflavones (49). The chemical structure of isoflavones is similar to mammalian estrogen due to the presence of a phenolic ring (49). This phenolic ring is a condition required for compounds that bind the estrogen receptor (ER) (30).

Isoflavones are found in high concentrations in soybeans and soy-derived proteins. The major isoflavones in soybeans are the isoflavone glycoside conjugates genistin and daidzin, and the isoflavone aglycones genistein and daidzein (58). Isoflavones have been discovered to have a broad range of hormonal and nonhormonal activities that aid in the prevention of hormonal-dependent diseases such as cancer (49). The isoflavone thought to be responsible for these activities is genistein. Genistein is believed to be responsible for anti-estrogenic and anti-carcinogenic activity in hormone dependent diseases (59, 67). However, for the most part, little is known about the mechanisms by which genistein performs these activities.

Genistein has been shown to be a specific inhibitor of tyrosine kinases. Akiyama, et al, observed genistein to inhibit epidermal growth factor (EGF) stimulated tyrosine phosphorylation of A431 cells. Further, it was seen that genistein competed with ATP and inhibition by genistein binding to enzyme-substrate complexes. This finding may explain why those cells lacking estrogen receptor are affected by genistein (2).

Genistein is believed to stimulate proliferation through estrogen receptor mediated pathways while its anti-proliferative actions are thought to be due to inhibition of tyrosine kinases (59). Two research studies have examined the anti-estrogenic effects of genistein on MCF-7 cells, a human breast cancer cell line that is positive for ER (59, 67). Both studies examined genistein's effects on ER binding, pS2 mRNA expression, pS2 protein, and cell proliferation. Wang, et al, (59) determined that genistein's effects were biphasic. At low doses, 10^{-8} to 10^{-5} M, genistein stimulated proliferation of MCF-7 but not an ER negative cell line (MDA-MB-231 cells). Genistein also stimulated pS2 mRNA expression and competed with estradiol to bind to ER in the MCF-7 cell line. At high doses, 2.5×10^{-5} to 10^{-4} M, genistein failed to stimulate MCF-7 pS2 mRNA expression and inhibited the proliferation of MCF-7 and the ER negative cell line. Wang, et al, (59), concluded that dietary genistein effects on hormone dependent diseases were ER dependent. This was because circulating genistein levels (62) would never reach levels seen with inhibition.

Zava and Duwe, (67), repeated the same procedures to examine genistein's estrogenic properties and antiproliferative activities. In addition, several other structurally similar iso- and bioflavonoids were used to compare with genistein's actions on MCF-7 cells. They concluded that genistein mediated ER dependent and independent activity over physiological concentration ranges (10 nM – 20 μ M) (67). The biphasic responses related to concentration were also noted. These studies suggested that genistein acts as an estrogen agonist at physiologic concentrations. However, at increased genistein concentrations, when proliferation was inhibited, the

pS2 protein was present suggesting an ER-independent cellular mechanism(s) may inhibit the ER-dependent proliferation (67).

Signal Transduction during NK Cell Activation

In cell biology, signal transduction is the process by which a cell converts an extracellular signal to produce intracellular signals to cause physiological responses. These responses can coordinate differentiation, development, cell maintenance, and metabolism (3). Signal transduction by ligand binding and/or biological response modifiers in NK cells results in proliferation, cytokine secretion, differentiation into LAK cells, and lytic activity.

Let's focus on those signaling events that mediate the cytotoxic function of NK cells by ADCC and natural cytotoxicity. Early and necessary events for these separate modes of killing include protein tyrosine kinase activation, release of phosphoinositides, and increase in free intracellular calcium (20, 42, 51). Cytolytic function mediated by ADCC is initiated by signaling through $Fc\gamma$ being bound by the Fc portion of antibodies bound to antigen. This receptor consists of a ligand-binding α subunit (CD 16) with noncovalently bound hetero- or homodimers of ξ (a component of TCR) and γ (a component of $Fc\gamma RI$) subunits. This multi-subunit receptor itself does not have intrinsic kinase activity but the ξ and γ subunits contain immunoreceptor tyrosine-based activation motifs (ITAMs) that aid in the generation of intracellular second messengers (9, 31). Upon binding of FcR , the ITAMs are phosphorylated by an intracellular PTK that enables the receptor to become coupled

with downstream signaling events. Two nonreceptor tyrosine kinase families, src (in NK cells Lck, Fyn, Yes, and Lyn) and syk (in NK cells Syk and ZAP-70), are thought to be responsible for this activity (9, 11, 31). In particular, the src-family kinase Lck has been linked to this phosphorylation activity since it has been found to physically associate with Fc γ RIII complex (17). Phosphorylation of ITAMs by Lck enables syk family kinases (ZAP-70 and Syk) to bind to them (11, 31). However, FcR complex signaling can occur in the absence of Lck, Fyn (another src-family kinase) also thought to couple with the receptor, and ZAP-70 (38, 57, 60). It has also been demonstrated that Syk activation (Lck independent) alone is sufficient to generate a cytotoxic response while ZAP-70 (Lck dependent) requires co-stimulation by a src family kinase (26). These findings prompted research looking into Syk kinase as the signaling element used to tyrosine phosphorylate ITAMs and initiate downstream signaling events. Through the use of pharmacological inhibition of Syk kinase activity (piceatannol, $\sim 6 \mu\text{g/ml}$), expression of dominant-negative kinase inactive Syk, and tumor cells transfected with MHC class I, it was determined that Syk kinase was an early and central signaling protein in ADCC and natural cytotoxicity (10).

Once the FcR complex gathers its activated kinases, it can activate a number of downstream signaling molecules. One of these substrates is phospholipase C- γ (PLC- γ) that is an enzyme that hydrolyzes phosphoinositides. The products from that reaction, inositol trisphosphate (IP3) and sn-1, 2-diacylglycerol (DAG), mediate the increase in free intracellular calcium and the activation of protein kinase C (PKC) (9). The increased concentration of free intracellular calcium is required for the release of granules in the delivery of the "lethal" hit (31).

Another downstream effector of FcR signaling is phosphatidylinositol 3-kinase (PI-3K). It was once thought that cytotoxic activities initiated through FcR ligation were PKC dependent since PKC-activating phorbol esters and calcium ionophores could induce NK cytotoxicity. However, it was shown that ADCC still occurred even in the presence of PKC inhibitors. It was found that natural lysis was PKC dependent while FcR lysis was PI-3K dependent (8).

Not only does the Fc-induced tyrosine kinase cascade activate downstream enzymes but it also activates small molecular weight GTP-binding proteins (G proteins). In the case of Ras, a G protein involved with mitogenic signaling pathways, it becomes activated by adapter proteins that have been phosphorylated by proximal PTKs induced by ligation of FcR. The adapter proteins p36 and Shc are tyrosine phosphorylated by these PTKs and then binds to another adapter protein, Grb2, which binds to Sos, the guanine nucleotide exchange factor for Ras. Sos stimulates inactive Ras to give bound GDP for GTP to become activated (9, 11). Ras activation is believed to regulate transcriptional events that mediate NK cell effector functions following receptor ligation (31). The guanine nucleotide exchange factor (GEF) Vav is tyrosine phosphorylated after FcR ligation after which it activates its downstream effector Rac1 (a small G protein). It has been shown that over expression of Vav enhances NK cell cytotoxicity and that a mutation that prevents Vav from mediating GTP for GDP on Rac1 will abolish this enhancement. Dominant-negative Rac1 has also been shown to inhibit NK cell mediated lysis. This all suggests that Rac1 is a downstream effector involved with regulation of cytotoxicity. This event is also observed with natural killing of target cells (6).

Signal transduction studies dealing with natural cytotoxicity have been hindered by a clear molecular characterization of a triggering receptor. Natural cytotoxicity like ADCC utilizes protein tyrosine kinase activation, PLC- γ release of phosphoinositides, and an increase in free intracellular calcium (20, 41, 51). Because natural cytotoxicity shares these early and requisite events with ADCC there was speculation that a receptor with ITAM subunits could be involved. Research has shown that if this is the case it would not be due to the ξ and γ since NK cells lacking these units can perform natural cytotoxicity (52). And like ADCC, Lck, Fyn, or ZAP-70 are not required for natural cytotoxicity (38, 57, 60). However, a central role for Syk tyrosine kinase was discovered in natural cytotoxicity (10). Natural cytotoxicity was found to be PKC dependent, PI-3K independent (8). Vav and its effector target Rac1 were found to have a role in regulating natural cytotoxicity (6). There is evidence that natural cytotoxicity can occur in the absence of PLC-dependent calcium signaling (43, 66).

Summary

Innate immunity is the first line of defense of the immune system. Natural killer cells play a role in innate immunity as effector cells. Tyrosine phosphorylation has been implicated as a signal for lytic activity of NK cells (20). In vitro studies with isoflavones, genistein and daidzein, have been shown to enhance NK lytic activity of peripheral blood leukocytes. This study was designed to ask if low concentrations of genistein had a direct effect on natural killer cell lytic activity and if this effect was

related to tyrosine phosphorylation. A pure NK cell line, NK3.3, a chromium release assay, and flow cytometry were used to study the direct effects of genistein on NK cells.

MATERIALS AND METHODS

Chemicals

Recombinant human Interleukin 2 (IL-2) (I-2644), Staurosporine A (S-4400), Saponin (S-7900), Sodium Orthovanadate (S-6508), Catalase (C-40), 3,3' - Diaminobenzidine (D-8001) were obtained from Sigma, St. Louis, MO. Formaldehyde, 16%, Ultra Pure, was obtained from Polysciences Inc., Warrington, PA. Sterile sodium chromate (^{51}Cr) obtained from NEN Dupont, Boston, MA. Genistein was synthesized in the laboratory of Dr. Patricia Murphy, Iowa State University, according to Chang et al (16). Genistein was analysed on a Beckman System Gold chromatography with an autosampler Model 507 (68).

Antibodies

Monoclonal anti-phosphotyrosine FITC- conjugate (F-3145) and mouse IgG1 κ (MOPC-21) FITC - conjugate (F-6397), monoclonal anti-phosphotyrosine (P-3300) and anti mouse IgG (Fab specific) peroxidase conjugate (A-2304) were obtained from Sigma, St. Louis, MO.

Isolation of NK Cells from Peripheral Blood

Blood was diluted 1:2 with washing solution. Washing solution was Hanks' Balanced Salt Solution (HBSS) and 25 mM Hepes (Gibco BRL, Grand Island, NY). Eight mls of diluted blood was slowly layered over 3 mls of Ficoll-Hypaque (Pharmacia Biotech, Piscataway, N.J.) in a 15 ml centrifuge tube. Blood samples

were centrifuged at 1500 rpm for 20 minutes at 20⁰ C. After centrifugation, plasma was pipetted and discarded. The buffy coat at the interface was collected by gently circling the centrifuge tube with a pipet. The buffy coat was then placed into 8 mls of washing solution and centrifuged at 1500 rpm for 5 minutes at 20⁰ C. Media was removed with just enough to cover resultant pellet of white blood cells. The pellet was resuspended by knocking tube on a hard surface. Eight mls of washing solution was added and the tube was centrifuged at 1500 rpm for 5 minutes at 20⁰ C. This step was repeated on more time but with complete media. After removing media and resuspending the pellet, 1.5 mls of complete media was added to the tube. Cell concentration of the sample was measured and test samples made from that concentration.

Cell Culture

NK3.3, a human cell line cloned from a primary mixed lymphocyte culture and phenotypically distinct from characterized PBL T-cell populations (27), were kindly provided by Dr. Jacki Kornbluth, St. Louis University, St. Louis, MO. NK3.3 cells were maintained in RPMI-1640, 2 mM L-glutamine, 25 mM Hepes, 50 ug/ml gentamicin (Gibco BRL, Grand Island, NY), 15% FBS (JRH, Biosciences, Lenexa, KS) and 15% Lymphocult-T (Biotest Diagnostics Corp, Denville, NJ) at 37⁰ C, 6% CO₂. Cell culture density was maintained at 2.5 - 3 X 10⁵ cells/ml.

K562 (ATCC, Rockville, MD), a myelogenous leukemia cell line, sensitive to NK cell lysis were maintained in RPMI-1640, 2 mM L-glutamine, 10 mM Hepes, 50 ug/ml gentamicin (Gibco BRL, Grand Island, NY), and 10% FBS (JRH, Biosciences,

Lenexa, KS) at 37⁰ C, 5% CO₂. Cell culture density was maintained at 3-9 X 10⁵ cells/ml.

Generation of Activated and Rested NK3.3 Cells

As NK3.3 cell activity varied as a result of IL-2 presence, cells were synchronized by activation with Lymphocult-T (the IL-2 source). NK3.3 cells were activated by resuspending cells in fresh media containing Lymphocult-T (15%) for 24 hours to bring all cells up to the same level of activity. Cells could be used after 18-20 hours for studies of “activated” NK3.3 cells. NK3.3 cells were separated into activated and rested media conditions after 24 hours stimulation with Lymphocult-T (15%). Cells defined as “activated” were resuspended again in fresh media containing Lymphocult-T for 18-20 hours prior to use in chromium release assays and flow cytometry methods. Cells defined as “rested” were resuspended in fresh media without Lymphocult-T for 18-20 hours prior to use in chromium release assays and flow cytometry methods.

NK3.3 Cell Stimulation and Inhibition

Human recombinant IL-2 was used in flow cytometry and chromium release assay at concentrations of 2.5, 5, 10, and 15 International Units (IU). Rested NK3.3 cells were incubated with IL-2 for 30 minutes at 37⁰ C, 5% CO₂ for the chromium release assay and for 10 or 30 minutes at 37⁰ C, 5% CO₂ for flow cytometry. IL-2 was used to restimulate NK 3.3 cells to respond to a natural ligand.

The following reagents were used in flow cytometry to manipulate intracellular tyrosine phosphorylation of NK3.3 cells. Sodium pervanadate (Na_3VO_4), a phosphatase inhibitor, was stored as a 50 mM vanadate solution in dH_2O . Prior to use, a vanadate solution of 5 mM was reacted with 1 mM H_2O_2 (equal portions) for 15 minutes at RT. Catalase (200 $\mu\text{g}/\text{ml}$) was added for 15 minutes at RT to remove residual H_2O_2 . The final concentration of pervanadate is denoted by final vanadate concentration. Staurosporine A was used as a tyrosine kinase inhibitor. A working concentration of 2 μM was used. NK3.3 cells were incubated with sodium pervanadate or staurosporine A for 10 minutes prior to staining for flow cytometry.

Genistein dilutions were made from a 10 mg/ml DMSO stock solution. Diluent used to make dilutions was a 2.5% DMSO/complete media mixture. Genistein was diluted and incubated with NK3.3 cells at final concentrations of 0, .1, .25, .5, 1, 2.5, 5, and 10 μM . Genistein dilutions used for flow cytometry were diluted with phosphate buffered saline (PBS)/2.5% DMSO. NK 3.3 cells were incubated with genistein for 30 minutes at 37°C , 5% CO_2 for the chromium release assay. NK3.3 cells used in flow cytometry were incubated with genistein for 10 and 30 minutes at 37°C , 5% CO_2 . The final concentration of DMSO was .025% and maintained constant across all dilutions for these assays. NK3.3 cells that were either incubated for 18-20 hours in fresh media with (activated) and without (rested) Lymphocult-T prior to use in cytotoxicity assays or fluorescent staining of intracellular phosphotyrosine residues.

Chromium Release Assay

The lytic ability of the NK 3.3 cells was measured by the amount of radioactivity released by K562 cells labeled with ^{51}Cr . Activated and rested NK3.3 cells were washed once and suspended in complete media at 2.85×10^6 cells/ml. K562 cells were incubated with 200 μCi of ^{51}Cr at 37°C , 5% CO_2 for 70 minutes. K562 cells were washed three times and suspended in complete media at 1.7×10^5 cells/ml. Effectors (NK3.3 cells) and target (K562) cells were plated in a E:T ratio of 50:1 in a final volume of 250 μl per well in a 96 well microtiter plate (Costar, Corning, NY). Spontaneous and total release controls were performed for each assay to establish natural release of ^{51}Cr (spontaneous) and maximum (total) release by complete lysis of K562 cells. All samples and controls were assayed in triplicate. The assay samples and controls were incubated at 37°C , 5% CO_2 for 3 hours. After incubation, 150 μl of 10% TCA was added to total control wells and the plate centrifuged at 500 rpm for 5 minutes (Jouan, Inc., CR3-21). One hundred μl of supernatant was removed from each well and placed in a one ml macrotubule (Phenix Research Products). Radioactivity was measured by a Gamma Trac 1191, (Tracor Analytic, Elk Grove Village, IL). The lytic activity of the NK3.3 cells was calculated using the formula:

$$\frac{\text{cpm experimental value} - \text{cpm spontaneous}}{\text{cpm total} - \text{cpm spontaneous}} \times 100 = \% \text{ cytotoxicity}$$

Spontaneous release was consistently < 10%.

Immunofluorescence Staining

A method for labeling intracellular phosphotyrosine residues was adapted from the methods of Farahi Far et al., (21) and Hubert et al., (24) for flow cytometry with NK3.3 cells. Activated and rested NK3.3 cells were washed once and suspended in PBS at a concentration of 5×10^6 cells/ml. One hundred μ l of cells were placed in an eppendorf tube with an equal volume of the following reagents: pervanadate, staurosporine A, IL-2, or genistein. Incubation time was for either 10 or 30 minutes. Treatment was stopped by washing NK3.3 cells with 600 μ l PBS/ Na_3N_3 /BSA (wash buffer) for 1 minute at 2000g in a microfuge. Samples were fixed with 200 μ l of 4% FMA for 10 minutes at 4⁰ C. Samples were then washed once with 200 μ l of 0.1 % permabilization solution (wash buffer containing saponin). NK3.3 cells were suspended in 100 μ l of permabilization solution and 7 μ l a 1:2 dilution of FITC-conjugated anti-phosphotyrosine antibody and incubated for 30 minutes on ice in the dark. Samples were washed twice with permabilization solution (200 μ l) and once with wash buffer (200 μ l). Samples resuspended in 1 ml of wash buffer prior to analysis by flow cytometry. Mean fluorescence was used as a parameter rather than % population to give better specificity for the results. Mean fluorescence is the average fluorescence of all cells in the sample.

Flow Cytometry Analysis

Analysis was performed on a XL-MCL (Coulter Corp., Miami, FL). Green fluorescence from FITC was collected through a 525 nm bandpass filter. Samples

were gated on forward scatter and side scatter (linear). Ten thousand gated events in a bit map were analyzed for each sample. Data was stored in a listmode file for reanalysis using Elite Software (Coulter Corp).

Statistical Analysis

A computerized program for analysis of variance (ANOVA, Statistix, NH Analytical Software) was used to assess differences between baseline and experimental values. ANOVA was used to examine the effects of 11 concentrations of genistein on activated NK3.3 cell lysis while a 2 X 11 ANOVA was used to examine the effects of genistein (11 concentrations) and IL-2 (-/+) on rested NK3.3 lysis. A 2 X 11 ANOVA was used to examine the effects of genistein (11 concentrations) and vanadate (-/+) on activated and rested NK3.3 cells intracellular tyrosine phosphorylation. The level of significance for the *F* test was set at $p < .05$.

RESULTS

Chromium Release Assays

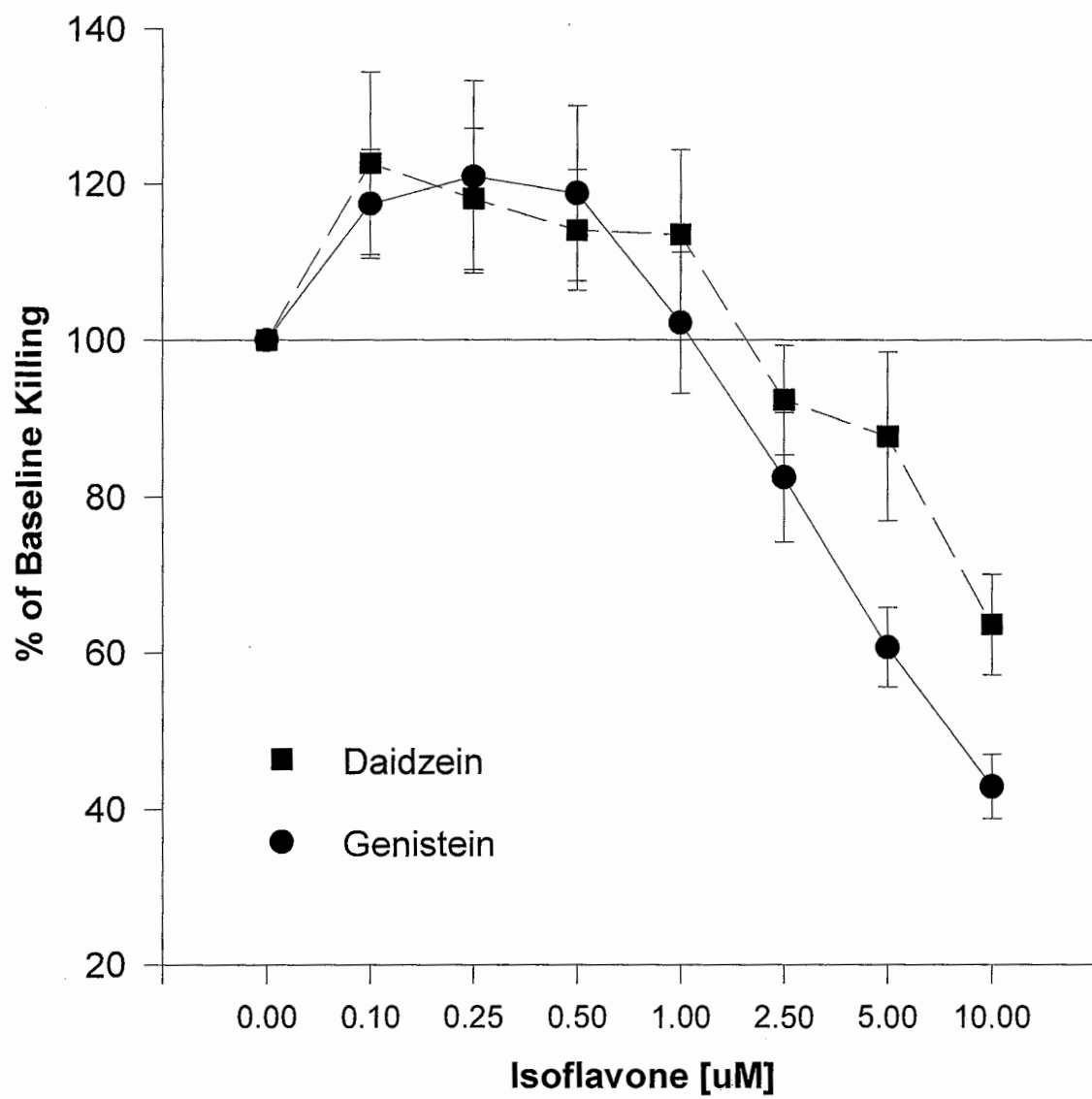
Studies with “NK-rich” populations

Chromium release assays were performed with NK cells harvested from peripheral blood leukocytes stimulated with genistein and daidzein. Figure 1 shows the average percent cytotoxicity of 8 chromium release assays with baseline set at 100% to standardize results. Baseline cytotoxicity for “NK-rich” samples varied between 13 to 58.8%. Lytic activity was 40% higher than that observed at baseline cytotoxicity. Lytic activity was significantly enhanced at concentrations 0.1 μ M to 1.0 μ M of daidzein and genistein ($p < .001$). Lytic activity was significantly suppressed at concentrations 2.5 μ M to 10.0 μ M. The amount of suppression due to these concentrations was found to be significantly greater with genistein ($p < .003$). We hypothesized that lytic enhancement was linked to tyrosine phosphorylation. As genistein was known to affect PTK's, we choose to study genistein in subsequent experiments. To see if enhancement of lytic activity was due to specific effects on NK cells, research with genistein was conducted on a NK cell line, NK3.3 cells (27).

Activated NK3.3 cells used to establish chromium release assay parameters

Activity of tumor killing by NK cells can be measured using a chromium release assay. However, the initial rate of lysis for NK3.3 is more rapid than that of freshly harvested peripheral NK cells and the 4.5-hour incubation regularly used for the chromium release assay did not yield the anticipated lytic activity. A kinetic study

Figure 1. Isoflavone enhancement of “NK-rich” cells. Genistein and daidzein enhanced lytic activity of NK cells harvested from peripheral blood. This enhancement was observed at .1 μ M to .5 μ M genistein with a 40% increase of lytic activity over baseline. The graph represents the average of 8 chromium release assays.

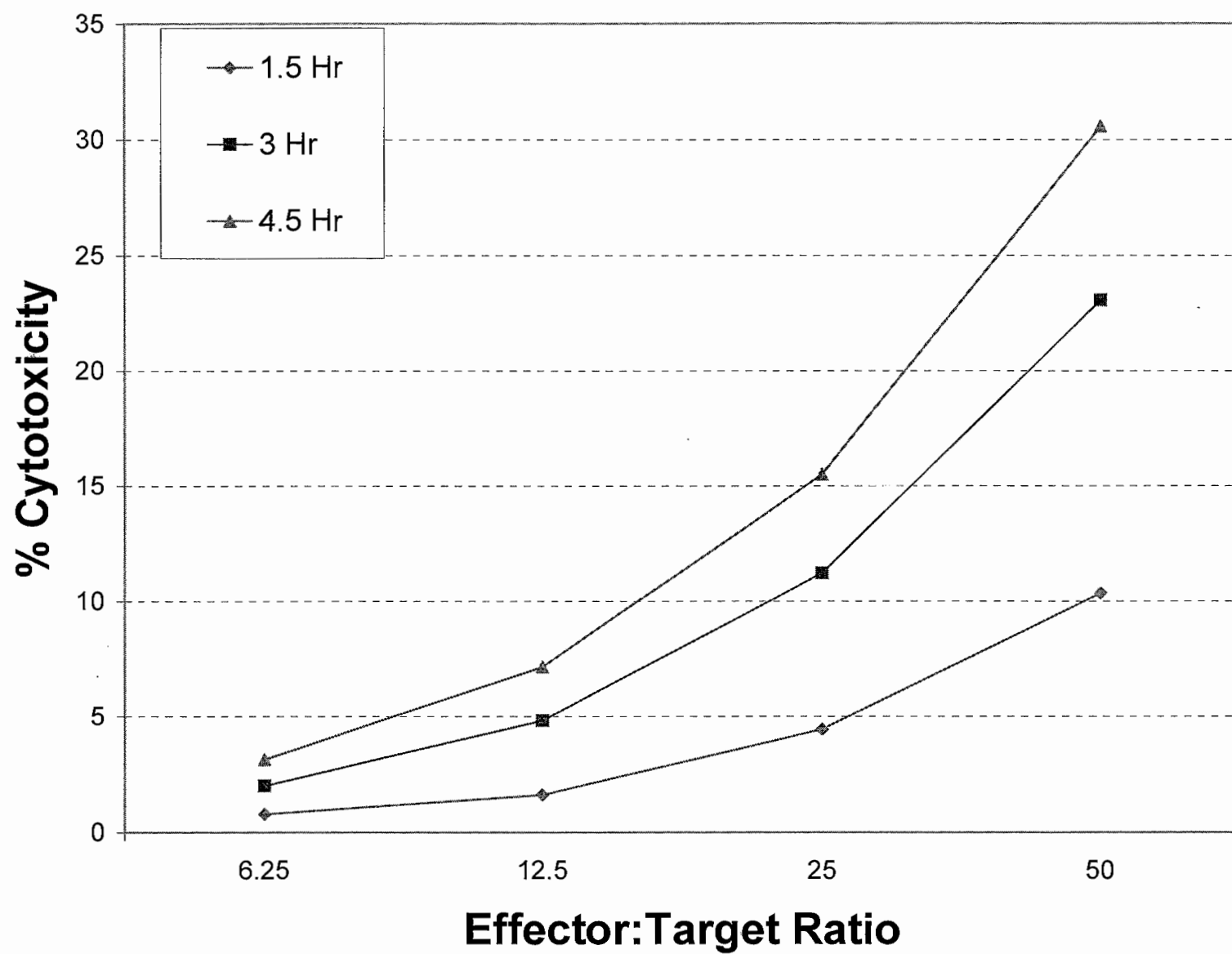


(33) with NK3.3 cells suggested NK3.3 cells could not readily recycle and lyse multiple target cells as well as freshly harvested peripheral NK cells. The study also determined that cytotoxic activity was also dependent on culture conditions before the assay and that the effector to target (E:T) cell ratio had no effect on cytotoxicity.

As culture conditions were found to have an effect on NK3.3 lytic activity (28), activated and rested cells were cultured in fresh IL-2 media for 24 hours to bring all cells up to the same level of activity before separation into activated and rested conditions. The “resting” NK3.3 cell condition was used to reproduce physiological conditions of peripheral NK cells.

Experiments were conducted with activated NK3.3 cells to define optimal cytotoxic assay conditions and the results are shown in figure 2. NK3.3 cells were incubated with radioactively labeled K562 cells for 1.5, 3, and 4.5 hours at effector to target ratios of: 6.25:1, 12.5:1, 25:1, and 50:1. The 4.5 hour incubation gave the highest amount of killing at all E:T ratios. However, the 3 hour incubation was chosen to examine the effects of genistein on NK3.3 lytic activity because the rate of killing appeared to slow between the 3 and 4.5 hour incubation times. The E:T (50:1) ratio also was chosen to use as it gave killing > 20%. It was found that rested NK3.3 cells did not yield significant cytotoxicity to evaluate optimal conditions. Therefore, the parameters established for activated cells were used for all other chromium release experiments performed with both activated and rested NK3.3 cells.

Figure 2. Activated NK3.3 cell lysis of K562 target cells at E:T ratios of 6:25 – 50:1 and incubation for 1.5, 3, and 4.5 hours. The graph represents the average of 4 chromium release assays for each incubation time/period.



Effects of IL-2 on rested NK3.3 cells

To determine if lytic function could be restored to rested NK3.3 cells, NK3.3 cells were incubated with IL-2 for 10 and 30 minutes prior to and during a 3-hour incubation with target cells. The results of IL-2 incubation with rested NK3.3 cells are shown in figure 3. Statistical analysis determined that incubation time was not a significant factor. IL-2 at 5 IU was not significantly different from 10 and 15 IU but significantly different from 0 ($p < .001$) and 2.5 IU ($p < .035$). This IL-2 concentration was used in conjunction with genistein for further lytic assays with rested NK3.3 cells because it was the lowest dose that gave the greatest increase in lytic activity.

Genistein's effects on lytic activity of activated and rested NK3.3 cells

To determine the direct effects of genistein on natural killer cells, activated and rested NK3.3 cells were incubated with genistein at concentrations of 0-10 μM for 30 minutes prior to adding chromium labeled K562 cells. These genistein concentrations were chosen because they cover the range of concentrations found in the plasma of people who eat soy-containing foods (62). Figure 4 shows the average cytotoxicity of activated NK3.3 cells incubated with genistein. Since the percent cytotoxicity values varied between chromium release assays but the trend remained the same, the baseline cytotoxicity of NK3.3 cells was set at 100% for each assay to normalize results. Baseline cytotoxicity for activated NK3.3 cells varied between 34.85% and 45.67%. Enhanced cytotoxicity of activated NK3.3 cells was observed at concentrations of 0.25 μM to 10 μM of genistein showing a significant effect of genistein compared to baseline killing ($p < .002$). The 0.1 μM

Figure 3. Rested NK3.3 cell lysis after incubation with IL-2. Rested NK3.3 cells were stimulated with IL-2 for either 10 or 30 minutes prior to a 3-hour incubation with K562 targets. Lytic function was enhanced when compared to baseline activity. Incubation time was found not to be a significant factor. IL-2 concentration at 5 IU ($p < .013$) was found to be the lowest dose that gave the greatest increase in lytic activity.

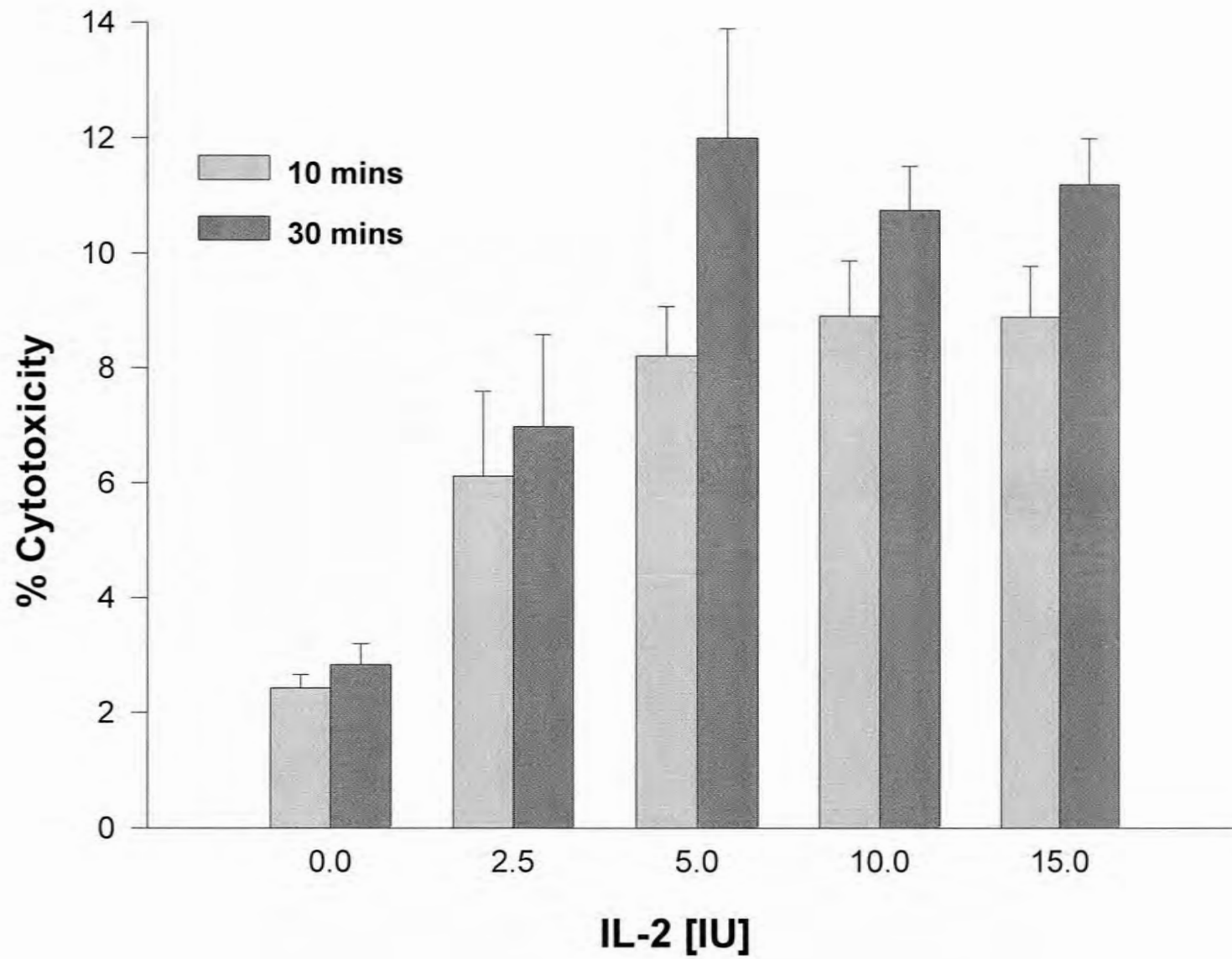
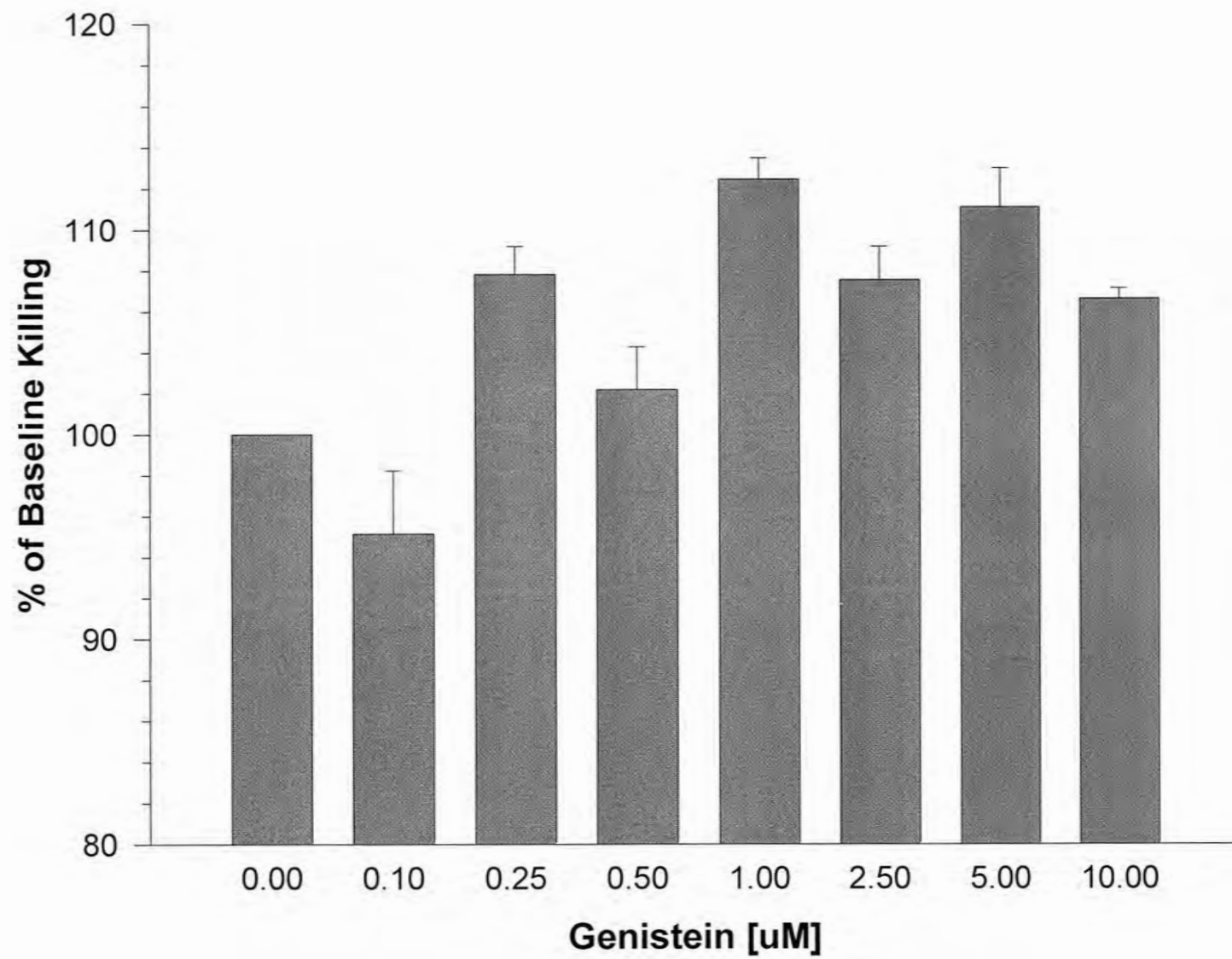


Figure 4. Genistein enhancement of activated NK3.3 lysis of K562 targets at a 50:1 ratio. Activated NK3.3 cells were stimulated with genistein 30 minutes prior to a 3-hour incubation with K562 targets. Baseline activity was set at 100% and experimental data was calculated relative to this control. Concentrations of 0.25 to 10.0 μ M genistein show an enhancement of lytic activity ($p < .002$). The graph represents an average of 4 chromium release assays.



concentration is not significantly different from baseline. But all other concentrations demonstrated significant enhancement compared to baseline (0) and ($p < .02$).

Figure 5 shows the average cytotoxicity for 4 experiments in which rested NK3.3 cells were incubated with genistein (Fig. 5A) or genistein plus IL-2 (5 IU/well; Fig. 5B). The baseline was set at 100% to normalize results. Baseline cytotoxicity for rested NK3.3 cells varied between 2.14 and 15.38%. Overall analysis of the effects of genistein and IL-2 showed significant enhancement by IL-2 across all genistein concentrations ($p < .0001$). There was marginally significant interaction of genistein and IL-2 ($p < .095$). This interaction indicates that genistein has a different effect on rested NK3.3 cells with or without IL-2.

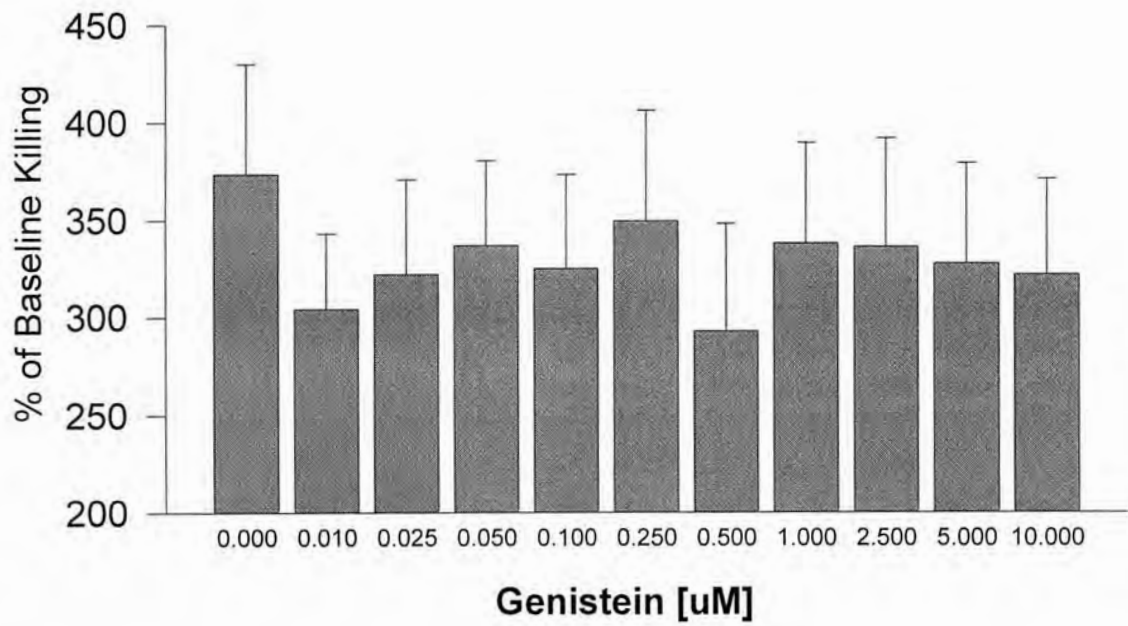
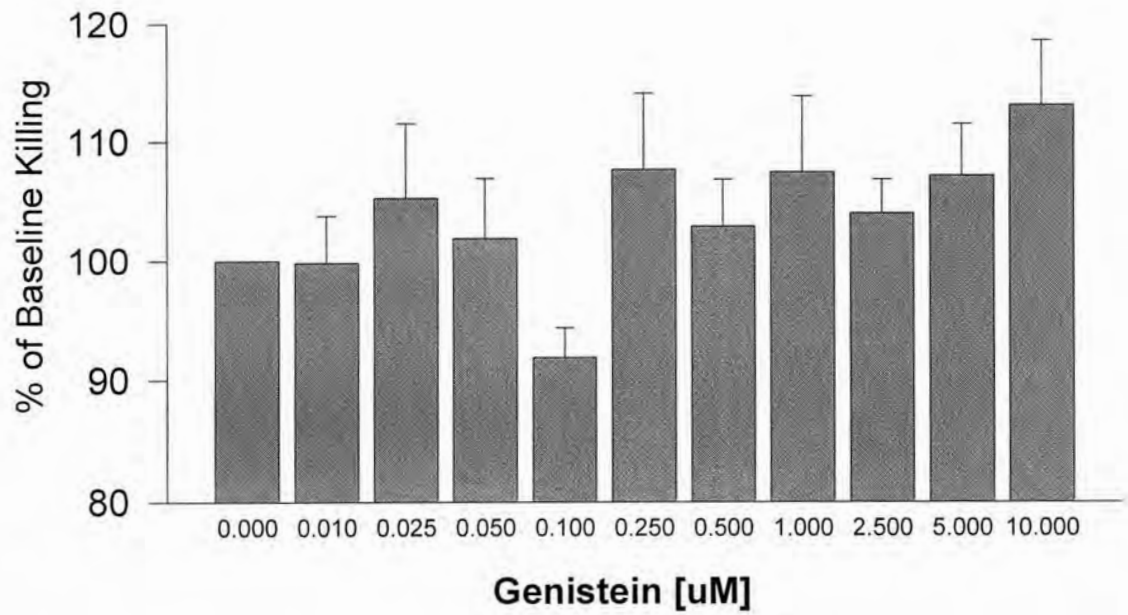
Rested NK3.3 cells incubated with genistein in the absence of IL-2 demonstrated enhanced cytotoxicity at concentrations of 1 to 10 μM of genistein with a range of 4 to 13%. Statistical analysis demonstrated a significant linear effect of genistein on enhancement of cytotoxicity. Specifically, genistein at concentrations of 1 to 10 μM enhanced cytotoxicity over the concentrations 0 to 0.1 μM . There was no significant difference between samples incubated with 0.01 to 0.1 μM .

Incubation with IL-2 increased cytotoxicity of rested NK3.3 cells by ~350% over baseline. However, rested NK3.3 cells stimulated with genistein and IL-2 showed significantly less cytotoxicity across all concentrations in comparison with NK3.3 cells incubated with IL-2 alone ($p < .007$). Thus, genistein inhibits IL-2 activation of resting NK3.3 cells.

Therefore, genistein alone is able to enhance cytotoxicity of activated and rested NK3.3 cells but inhibits the activation of rested NK3.3 cells by IL-2.

Figure 5. Rested NK3.3 treated with genistein (A) or with genistein and IL-2 (B).

Lysis of K562 targets at a 50:1 ratio was enhanced at concentrations of 1 to 10 μ M ($p < .0076$). The graph represents the average of 4 chromium release assays. Cytotoxicity of rested NK3.3 cells without genistein or IL-2 was set at 100% and experimental data was calculated relative to this control (A). Lysis of K562 targets at a 50:1 ratio was inhibited across all concentrations of genistein in comparison to IL-2 control cells (0 genistein) ($p < .0068$). The graph represents the average of 4 chromium release assays. Cytotoxicity was calculated relative to the 100% cytotoxicity established for rested NK3.3 cells without genistein or IL-2 (B).



Flow Cytometry

Quantitation of Intracellular Phosphotyrosine

Activation of NK cell cytotoxicity is dependent on tyrosine phosphorylation (20). Intracellular phosphorylated tyrosine residues can be stained by antibody conjugated with fluorescent dye and then analyzed by flow cytometry. The level of tyrosine phosphorylation is the resultant balance between phosphorylation by tyrosine kinases and dephosphorylation by tyrosine phosphatases. Specific inhibitors of these enzymes can experimentally regulate intracellular tyrosine phosphorylation levels of NK3.3 cells. Vanadate, a tyrosine phosphatase inhibitor, and staurosporine A, a protein tyrosine kinase inhibitor, were used to modulate tyrosine phosphorylation levels in NK3.3 cells.

These modulators allowed for amplification and inhibition of intracellular phosphotyrosine levels and allowed for a comparison to be made on intracellular phosphotyrosine levels of NK3.3 cells stimulated with genistein. Vanadate (200 μ M) and staurosporine A (200 nM) also served as positive and negative controls for flow cytometry. Figure 6 is a histogram demonstrating the extent to which these modulators regulate phosphotyrosine levels of NK3.3 cells in comparison to baseline phosphorylation.

Vanadate or staurosporine A was incubated with rested and activated NK3.3 cells to alter intracellular phosphotyrosine levels prior to analysis by flow cytometry. Figures 7A and 7B demonstrates the mean fluorescence intensity of cells labeled with fluorochrome tagged anti-phosphotyrosine antibody after incubation with vanadate or staurosporine A. Statistical analysis for vanadate showed a significant

Figure 6. A comparison of intracellular phosphotyrosine levels of NK3.3 cells incubated with and without vanadate and staurosporine A. The histogram displayed demonstrates the mean fluorescence intensity of NK3.3 cells stained with FITC conjugated anti-phosphotyrosine antibody. NK3.3 cells were treated with 200 μ M of vanadate (green), 200 nM of staurosporine A (red), or control media (baseline, blue) and analysed by flow cytometry for intracellular phosphotyrosine levels.

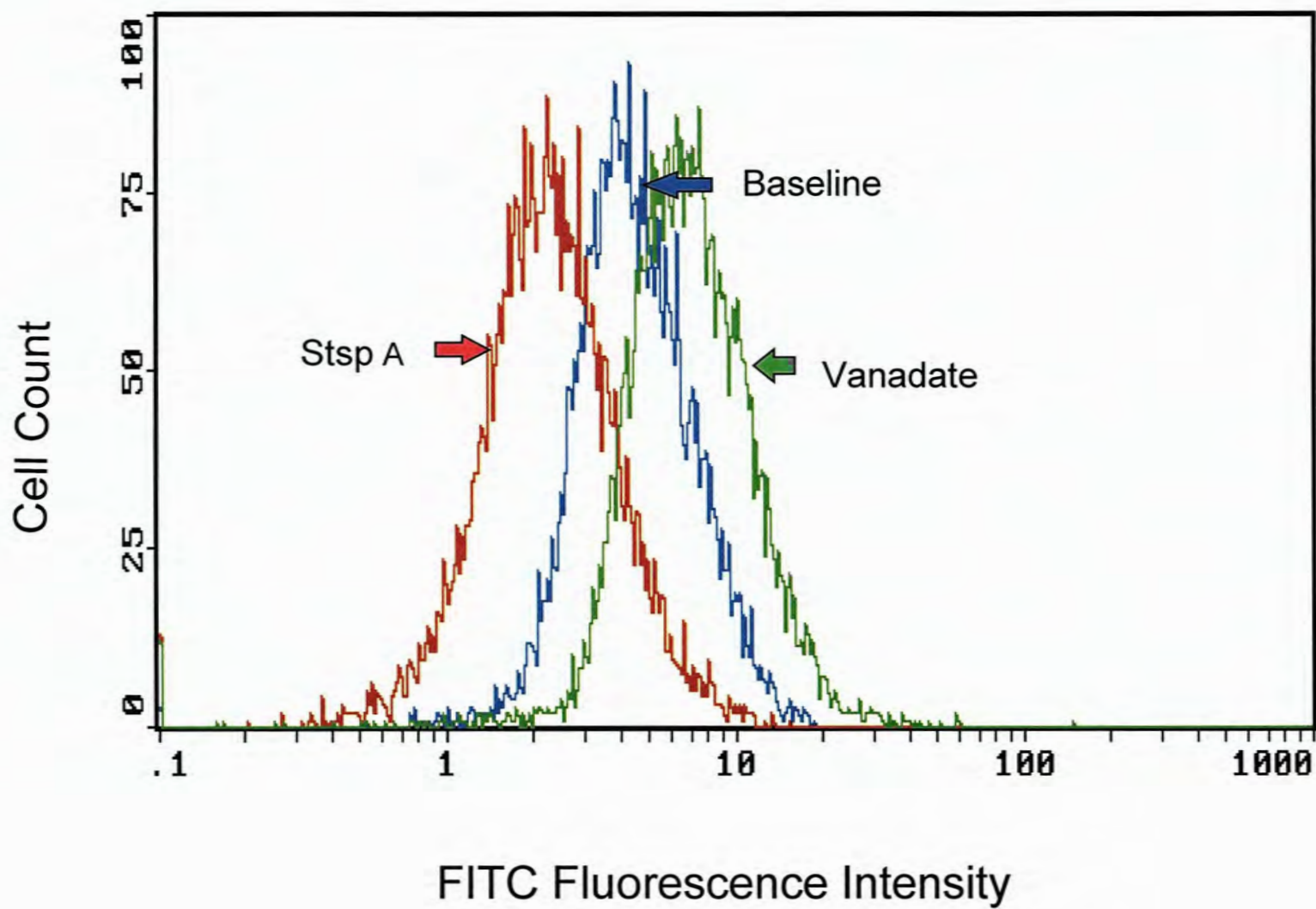
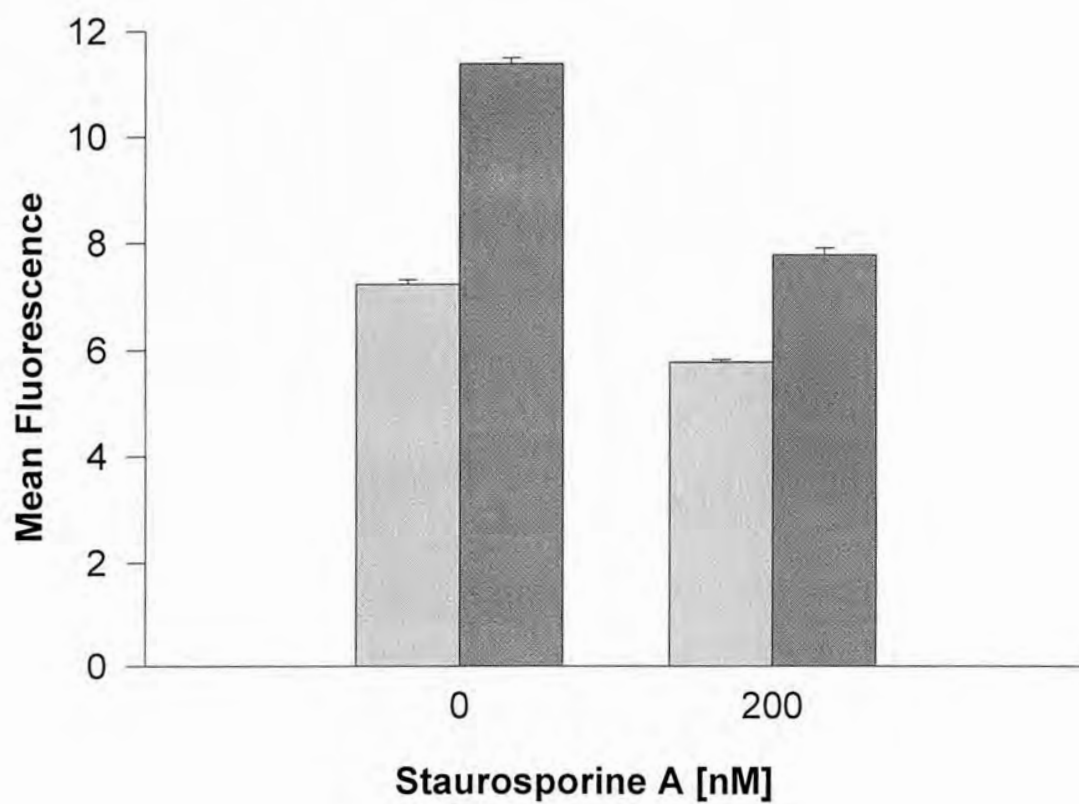
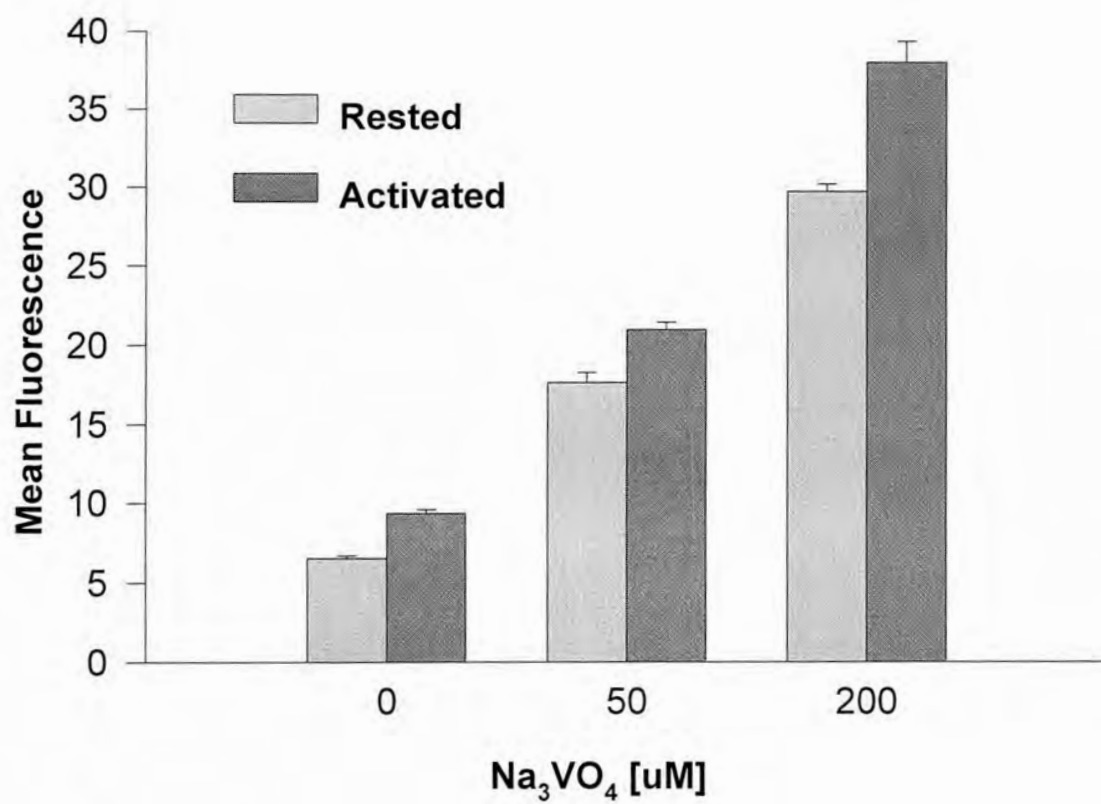


Figure 7. Intracellular phosphotyrosine levels of rested and activated NK3.3 cells stimulated with vanadate (A) or suppressed with staurosporine A (B).

Intracellular phosphotyrosine levels are increased by incubation with 50 μ M and 200 μ M concentrations of vanadate in contrast to levels seen with 0 μ M/baseline ($p < .001$) (A). Intracellular phosphotyrosine levels are decreased upon exposure to 200 nM of staurosporine A in comparison to levels observed at 0 μ M/baseline ($p < .001$) Staurosporine A suppressed activated NK3.3 cells to a greater degree than rested cells ($p < .003$) (B).

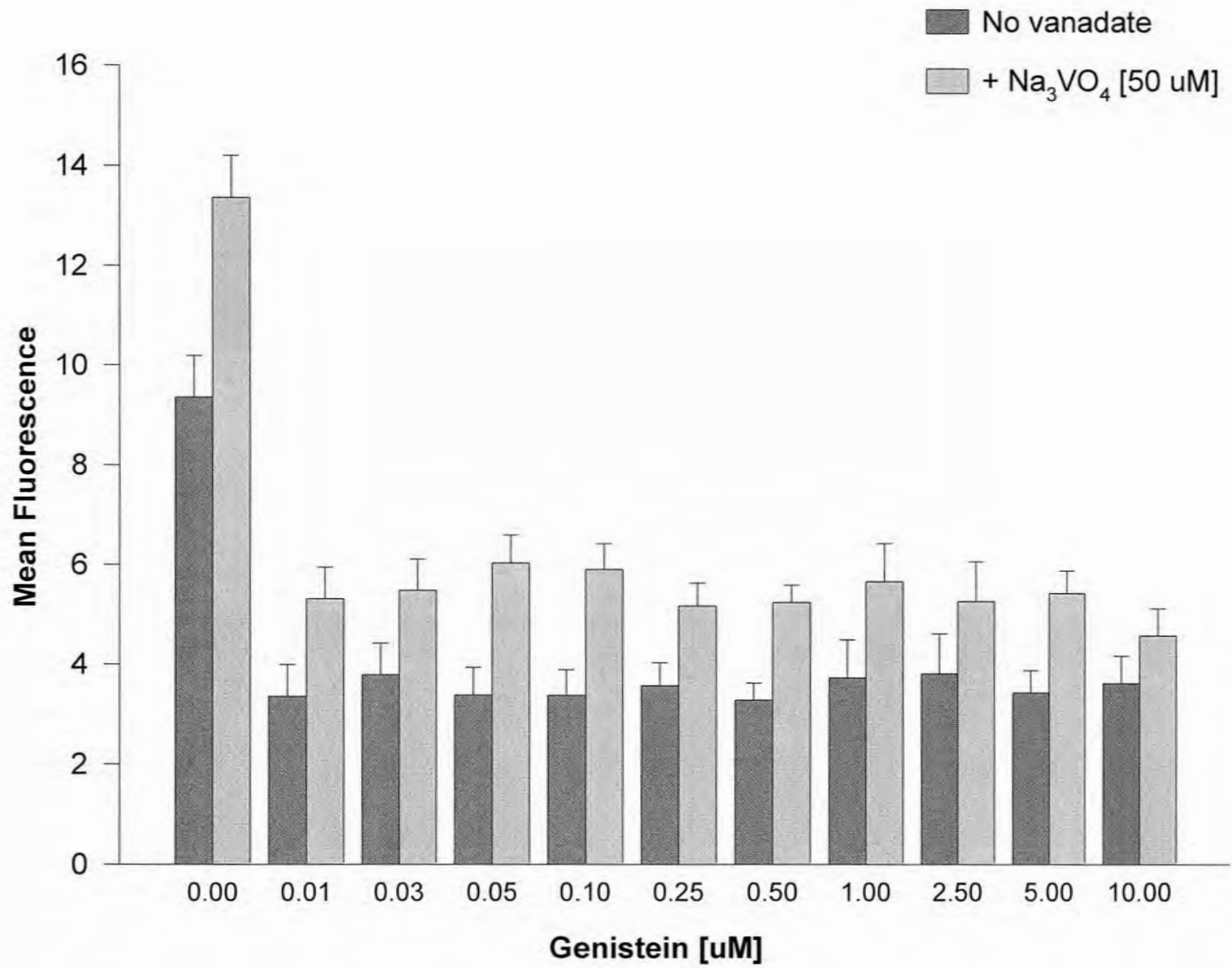


difference between concentrations ($p < .001$) and between rested and activated NK3.3 cells ($p < .003$). Statistical analysis showed a significant difference between baseline and suppression by staurosporine A ($p < .001$), between rested and activated NK3.3 cells ($p < .001$), and that staurosporine A suppressed activated NK3.3 cells to a greater degree than rested ($p < .003$). Mean fluorescent intensity was used rather than percent positive because stained cell populations shifted up or down (as seen in figure 6) rather than separating into distinct populations.

Intracellular phosphotyrosine levels of activated NK3.3 cells with genistein

To determine if there was a correlation between the concentrations of genistein that enhanced lytic activity and intracellular phosphotyrosine levels, flow cytometry analysis was performed on activated NK3.3 cells after a 10-minute incubation with genistein or genistein plus vanadate ($50 \mu\text{M}$). Incubation in the presence of vanadate was done to amplify any changes in tyrosine phosphorylation due to incubation with the low doses of genistein. (Vanadate at $50 \mu\text{M}$ was used as it was found to be the concentration for optimal amplification as shown in figure 7). Figure 8 shows the mean fluorescent intensity of activated NK3.3 cells stained for intracellular phosphotyrosine after incubation in the presence or absence of vanadate and genistein concentrations in a range of 0 to $10 \mu\text{M}$. Genistein was seen to suppress intracellular phosphotyrosine levels across all concentrations ($p < .002$). There was no difference in suppression among the samples incubated with genistein. Vanadate enhanced tyrosine phosphorylation across all concentrations of genistein ($p < .003$). However, there was no significant interaction of genistein and vanadate indicating

Figure 8. Intracellular phosphotyrosine levels of activated NK3.3 cells incubated with genistein and genistein plus 50 μ M of vanadate. Activated NK3.3 cells were incubated for 10 minutes with either genistein or genistein plus vanadate prior to staining with FITC-conjugated antiphosphotyrosine antibody for flow cytometry analysis. Genistein across all concentrations suppressed intracellular phosphotyrosine levels, even with the presence of 50 μ M vanadate ($p < .002$). Vanadate enhanced intracellular tyrosine phosphorylation ($p < .003$). There was no significant interaction between genistein and vanadate.

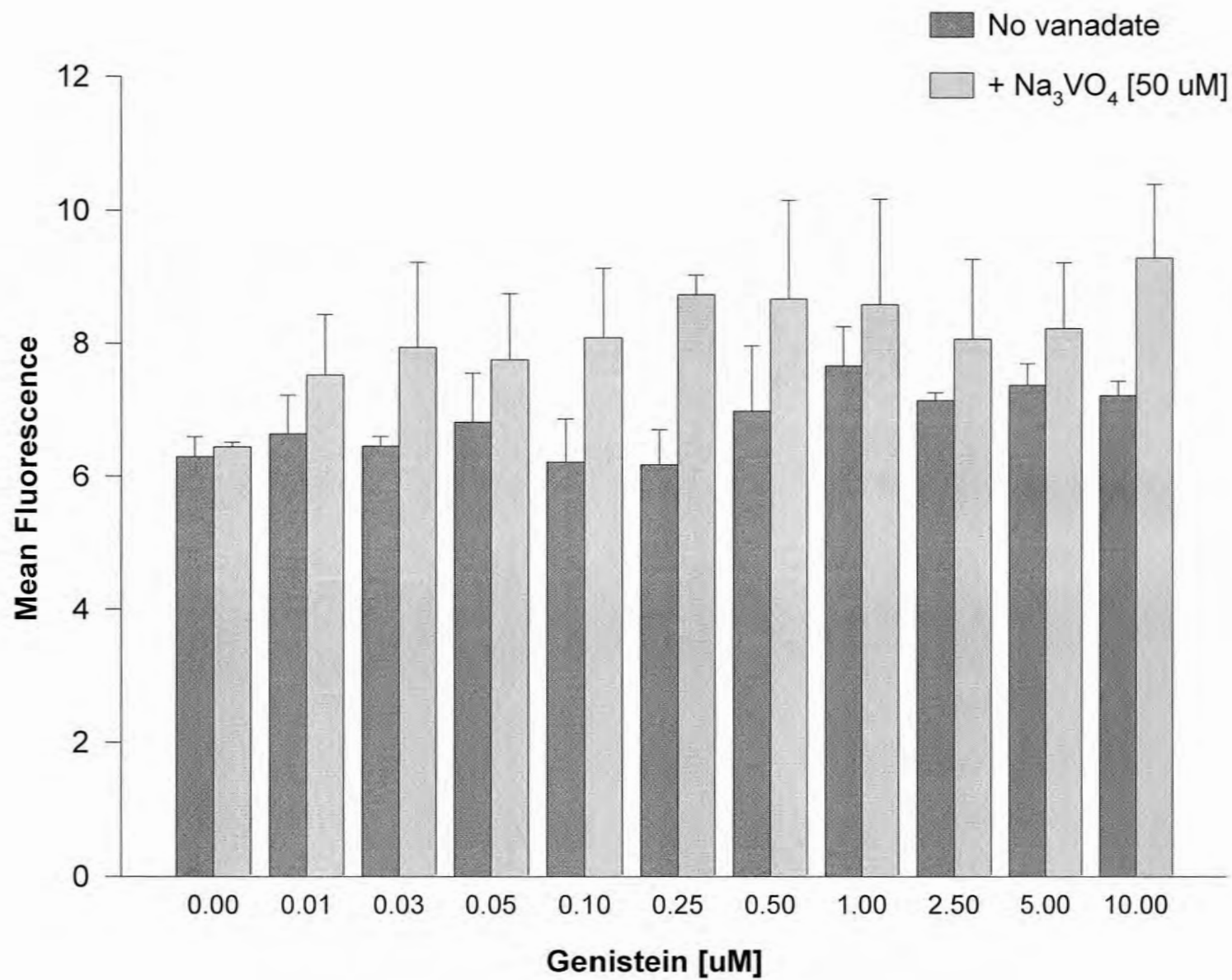


that the amount of suppression due to genistein was the same in samples with or without vanadate. In addition, this data supports the premise that vanadate can amplify changes in tyrosine phosphorylation without affecting the trend of the effect.

Intracellular phosphotyrosine levels of rested NK3.3 cells with genistein

To see if there was a difference between activated and rested intracellular phosphotyrosine levels, rested NK3.3 cells were incubated with genistein or genistein plus vanadate (50 μ M) for 10 minutes prior to staining and flow cytometric analysis. Figure 9 shows that in the case of rested NK3.3 cells genistein significantly enhanced intracellular phosphotyrosine levels ($p < .003$). Vanadate increased the levels of intracellular phosphotyrosine ($p < .0001$). LSD (least significant difference) comparison of means demonstrated that intracellular phosphotyrosine levels were enhanced across all concentrations of genistein in comparison to baseline ($p < .05$). However, there was no significant interaction of genistein and vanadate. This indicates that vanadate amplified the effects of genistein on intracellular phosphotyrosine, which made the effects of genistein easier to observe.

Figure 9. Intracellular phosphotyrosine levels of rested NK3.3 cells incubated with genistein and genistein plus 50 μ M of vanadate. Rested NK3.3 cells were incubated for 10 minutes with either genistein or genistein plus vanadate prior to staining with FITC-conjugated phosphotyrosine antibody for flow cytometry analysis. Genistein significantly enhanced intracellular phosphotyrosine levels across all concentrations ($p < .005$). This enhancement was also observed when tyrosine phosphorylation was amplified with vanadate.



DISCUSSION

Our studies with NK cells isolated from human peripheral blood showed enhancement of lytic activity in the presence of low doses of genistein and daidzein. This enhancement was observed specifically at concentrations of 0.1 μ M to 0.5 μ M with lytic activity 40% higher than baseline cytotoxicity. This is the first time that daidzein has been shown to enhance lytic activity like genistein.

Genistein was studied because a great deal of literature has been written about it being a PTK inhibitor. We were interested to see if this enhancement of lytic activity was linked to tyrosine phosphorylation.

It was unclear from these results if genistein was having a direct or indirect effect on peripheral blood NK cells. Thus, a pure NK cell population was needed to examine the direct effects of genistein on cytotoxicity and intracellular phosphotyrosine. The NK3.3 cell line was used to answer these questions.

The NK3.3 cell line solved the problems of lack of quantity and a pure cell population. However, it is a cloned cell line, which means differences could exist from NK cells harvested from fresh peripheral blood leukocytes. NK3.3 cell growth is dependent on the continued presence of IL-2. This is not known about the physiological environment for peripheral NK cells. IL-2 in the media maintains the lytic activity of NK3.3 cells but peripheral NK cells are also constitutively active.

Genistein enhances lytic activity of peripheral blood NK cells in the presence and absence of IL-2 (68).

This research sought to correlate the enhancement of NK3.3 cytotoxicity as determined by chromium release assays by low doses of genistein with changes in protein tyrosine kinase activity determined by flow cytometry analysis of intracellular phosphotyrosine residues. In the case of activated NK3.3 cells, genistein increased cytotoxicity but suppressed intracellular phosphotyrosine levels in parallel samples. However, with rested NK3.3 cells a correlation was observed between genistein enhancement of cytotoxicity and intracellular phosphotyrosine at the higher concentrations tested (0.25 – 10 μ M).

Genistein was observed to enhance cytotoxicity of activated NK3.3 cells at concentrations of 0.25 to 10 μ M in chromium release assays by ~ 14%. This reflects a broader range of enhancement of lytic activity than the studies using “NK-rich” cell populations of peripheral blood lymphocytes. Those studies demonstrated no enhancement at 1 μ M and suppression at doses > 5 μ M. Genistein was seen to suppress phosphorylation of tyrosine residues at all concentrations as detected by flow cytometry analysis. This suggests that genistein enhancement of cytotoxicity can act through a pathway other than PTKs.

Experiments using rested NK3.3 cells, which were designed to mimic the physiological state of peripheral NK cells harvested from blood, not only showed enhancement of cytotoxicity, but also had a corresponding enhancement of intracellular phosphotyrosine residues. Genistein at concentrations of 0.25 to 10 μ M enhanced cytotoxicity by ~ 13% as determined by chromium release assays. Again

this concentration range differs from that observed with “NK-rich” population studies. Genistein was seen to enhance intracellular phosphotyrosine levels of rested NK3.3 cells across all concentrations. This enhancement was amplified by the presence of vanadate.

Taking all results together, the enhancement of NK3.3 cytotoxicity by genistein appears to be acting through a pathway independent of tyrosine phosphorylation. Baseline intracellular phosphotyrosine for rested NK3.3 cells is lower than that observed for activated NK3.3 cells. The effect of genistein on NK3.3 cell PTK activity is clearly dependent on the activation state of the cell.

Though rested NK3.3 cells could have lytic activity restored by IL-2, genistein was found to suppress this effect. The increase in cytotoxicity of rested NK3.3 cells due to genistein was less than that when stimulated with IL-2. In addition, incubation of rested NK3.3 cells with IL-2 and genistein demonstrated less cytotoxicity than incubation with IL-2 alone. This would suggest that genistein enhancement is not through the use of IL-2 receptors and/or that it is using a pathway separate from IL-2 activation. Alternatively, different receptors maybe expressed that genistein can act upon depending on the activation state of the cell.

Genistein at low doses on NK3.3 cells showed an enhancement of lytic activity over a broader concentration range than that of “NK-rich” populations. However, this lytic activity was not as great as that seen with the “NK-rich” populations, suggesting that genistein is also acting upon other cell types in the population, which could lead to cross talk between those cells and NK cells.

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ACKNOWLEDGMENTS

I would like to thank the four greatest experiments in my life. Kyle Michael, Jared Matthew, Joel Patrick, and Emily Louise. Without my kids and wanting to show them that anything is possible and how important an education is, I would never have been able to make it through the struggles and frustration. Thank you for forgiving me the missed soccer games, swim meets, and school events. I promise you that our lives will be the richer from now on!

I would like to thank my grandfather, Roy Madison Casper, for always believing in me and making my graduate education possible. My mother, Patricia Ann Casper, for teaching me the importance of an education and showing me how a strong woman survives. My husband, Dr. James Michael Downard, for believing in me.